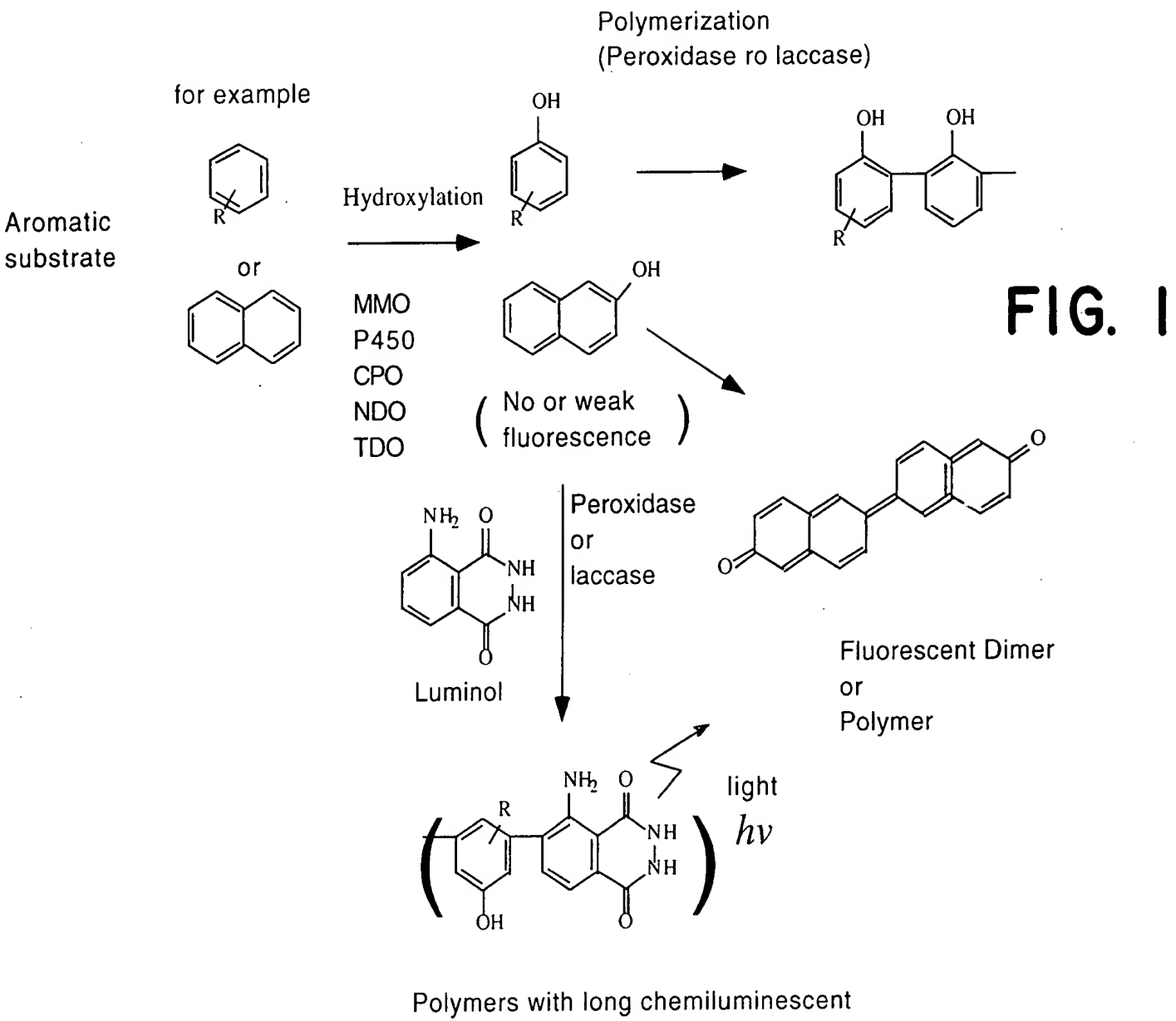

Polymerization
(Peroxidase ro laccase)
for example
Aromatic substrate
Hydroxylation
or
MMO
P450
CPO
NDO
TDO
OH
R
OH
OH
R
OH
( No or weak fluorescence )
Peroxidase
or
laccase
NH2
O
NH
NH
O
Luminol
O
O
Fluorescent Dimer
or
Polymer
NH2
O
R
NH
NH
OH
O
light
hv
Polymers with long chemiluminescent

FIG. 1

FIG. 2

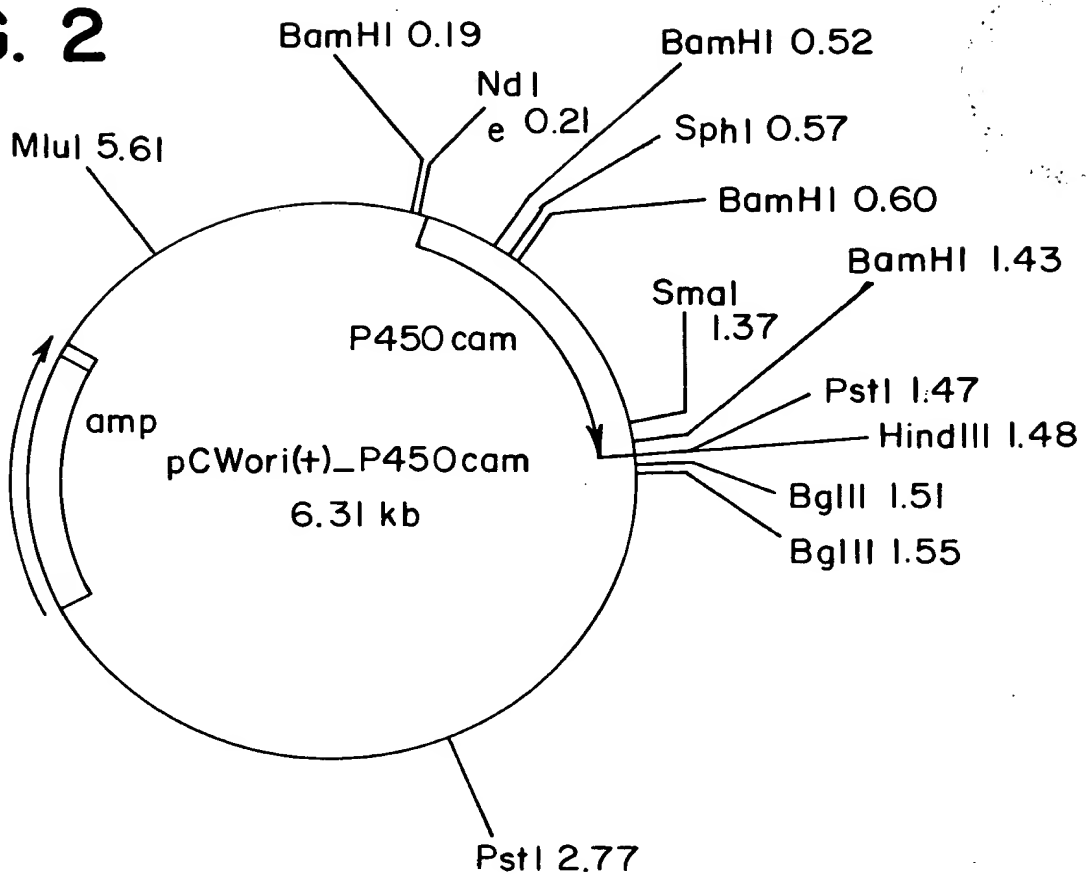
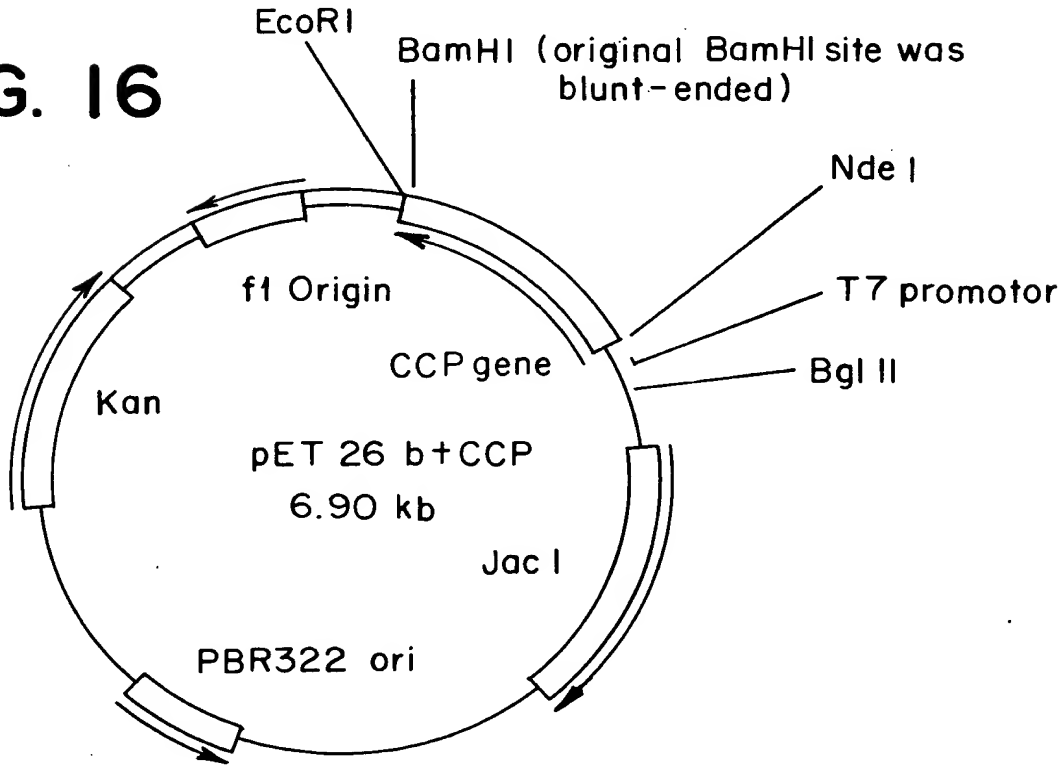

BamHI 0.19
BamHI 0.52
Nd I
e 0.21
Sph I 0.57
MluI 5.61
BamHI 0.60
BamHI 1.43
SmaI
1.37
P450cam
PstI 1.47
amp
HindIII 1.48
pCWori(+)_P450cam
6.31 kb
BglII 1.51
BglII 1.55
PstI 2.77

FIG. 16


EcoRI
BamHI (original BamHI site was
blunt-ended)
Nde I
T7 promotor
Bgl II
f1 Origin
CCP gene
Kan
pET 26 b+CCP
6.90 kb
Jac I
PBR322 ori

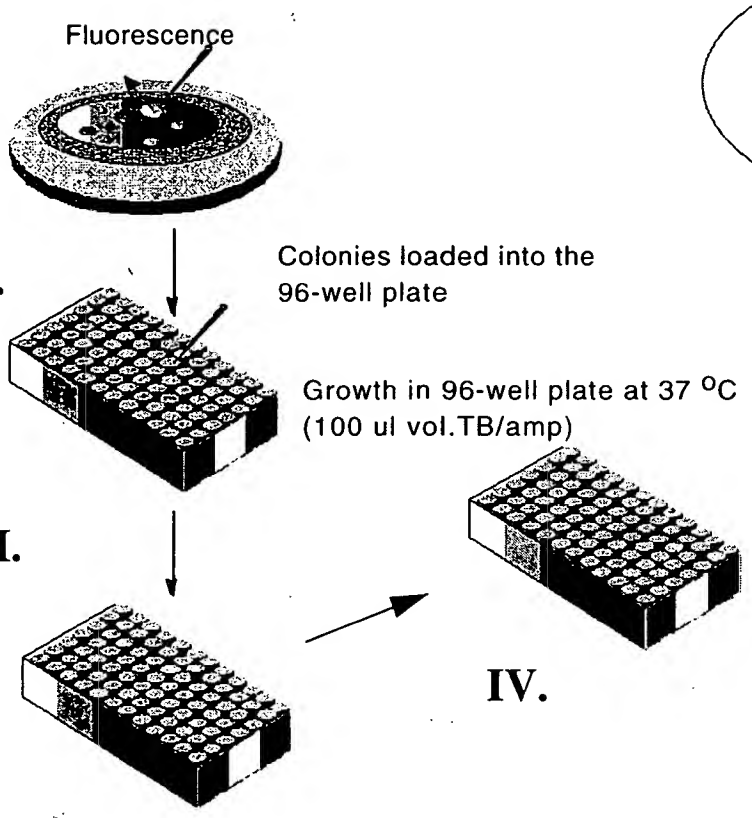
# FIG. 3A

```
   1 CTGCAGGATC GTTATCCGCT GGCCGATCTG ATCACCCAGC GTTTTTCCAT CGACGAGGCC
  61 AGCAAGGCAC TTGAACTGGT CAAGGCAGGA GCACTGATCA AACCCGTGAT CGACTCCACT
 121 CTTTAGCCAA CCCGCGTTCC AGGAGAACAA CAACAATGAC GACTGAAACC ATACAAAGCA
 181 ACGCCAATCT TGCCCCTCTG CCACCCCATG TGCCAGAGCA CCTGGTATTC GACTTCGACA
 241 TGTACAATCC GTCGAATCTG TCTGCCGGCG TGCAGGAGGC CTGGGCAGTT CTGCAAGAAT
 301 CAAACGTACC GGATCTGGTG TGGACTCGCT GCAACGGCGG ACACTGGATC GCCACTCGCG
 361 GCCAACTGAT CCGTGAGGCC TATGAAGATT ACCGCCACTT TTCCAGCGAG TGCCCGTTCA
 421 TCCCTCGTGA AGCCGGCGAA GCCTACGACT TCATTCCCAC CTCGATGGAT CCGCCCGAGC
 481 AGCGCCAGTT TCGTGCGCTG GCCAACCAAG TGGTTGGCAT GCCGGTGGTG GATAAGCTGG
 541 AGAACCGGAT CCAGGAGCTG GCCTGCTCGC TGATCGAGAG CCTGCGCCCG CAAGGACAGT
 601 GCAACTTCAC CGAGGACTAC GCCGAACCCT TCCCGATACG CATCTTCATG CTGCTCGCAG
 661 GTCTACCGGA AGAAGATATC CCGCACTTGA AATACCTAAC GGATCAGATG ACCCGTCCGG
 721 ATGGCAGCAT GACCTTCGCA GAGGCCAAGG AGGCGCTCTA CGACTATCTG ATACCGATCA
 781 TCGAGCAACG CAGGCAGAAG CCGGGAACCG ACGCTATCAG CATCGTTGCC AACGGCCAGG
 841 TCAATGGGCG ACCGATCACC AGTGACGAAG CCAAGAGGAT GTGTGGCCTG TTACTGGTCG
 901 GCGGCCTGGA TACGGTGGTC AATTTCCTCA GCTTCAGCAT GGAGTTCCTG GCCAAAAGCC
 961 CGGAGCATCG CCAGGAGCTG ATCGAGCGTC CGGAGCGTAT TCCAGCCGCT TGCGAGGAAC
1021 TACTCCGGCG CTTCTCGCTG GTTGCCGATG GCCGCATCCT CACCTCCGAT TACGAGTTTC
1081 ATGGCGTGCA ACTGAAGAAA GGTGACCAGA TCCTGCTACC GCAGATGCTG TCTGGCCTGG
1141 ATGAGCGCGA AAACGCCTGC CCGATGCACG TCGACTTCAG TCGCCAAAAG GTTTCACACA
1201 CCACCTTTGG CCACGGCAGC CATCTGTGCC TTGGCCAGCA CCTGGCCCGC CGGGAAATCA
1261 TCGTCACCCT CAAGGAATGG CTGACCAGGA TTCCTGACTT CTCCATTGCC CCGGGTGCCC
1321 AGATTCAGCA CAAGAGCGGC ATCGTCAGCG GCGTGCAGGC ACTCCCTCTG GTCTGGGATC
1381 CGGCGACTAC CAAAGCGGTA TA
```

# FIG. 3B

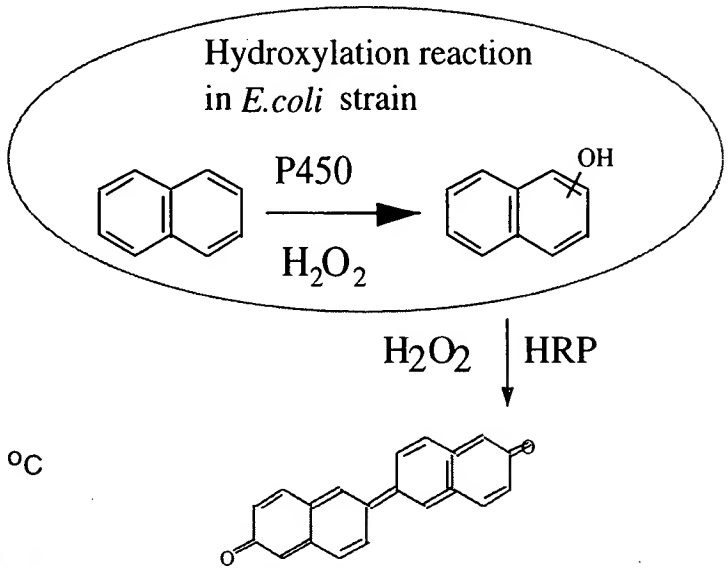
THR THR GLU THR ILE GLN SER ASN ALA ASN LEU ALA PRO
LEU PRO PRO HIS VAL PRO GLU HIS LEU VAL PHE ASP PHE
ASP MET TYR ASN PRO SER ASN LEU SER ALA GLY VAL GLN
GLU ALA TRP ALA VAL LEU GLN GLU SER ASN VAL PRO ASP
LEU VAL TRP THR ARG CYS ASN GLY GLY HIS TRP ILE ALA
THR ARG GLY GLN LEU ILE ARG GLU ALA TYR GLU ASP TYR
ARG HIS PHE SER SER GLU CYS PRO PHE ILE PRO ARG GLU
ALA GLY GLU ALA TYR ASP PHE ILE PRO THR SER MET ASP
PRO PRO GLU GLN ARG GLN PHE ARG ALA LEU ALA ASN GLN
VAL VAL GLY MET PRO VAL VAL ASP LYS LEU GLU ASN ARG
ILE GLN GLU LEU ALA CYS SER LEU ILE GLU SER LEU ARG
PRO GLN GLY GLN CYS ASN PHE THR GLU ASP TYR ALA GLU
PRO PHE PRO ILE ARG ILE PHE MET LEU LEU ALA GLY LEU
PRO GLU GLU ASP ILE PRO HIS LEU LYS TYR LEU THR ASP
GLN MET THR ARG PRO ASP GLY SER MET THR PHE ALA GLU
ALA LYS GLU ALA LEU TYR ASP TYR LEU ILE PRO ILE ILE
GLU GLN ARG ARG GLN LYS PRO GLY THR ASP ALA ILE SER
ILE VAL ALA ASN GLY GLN VAL ASN GLY ARG PRO ILE THR
SER ASP GLU ALA LYS ARG MET CYS GLY LEU LEU LEU VAL
GLY GLY LEU ASP THR VAL VAL ASN PHE LEU SER PHE SER
MET GLU PHE LEU ALA LYS SER PRO GLU HIS ARG GLN GLU
LEU ILE GLU ARG PRO GLU ARG ILE PRO ALA ALA CYS GLU
GLU LEU LEU ARG ARG PHE SER LEU VAL ALA ASP GLY ARG
ILE LEU THR SER ASP TYR GLU PHE HIS GLY VAL GLN LEU
LYS LYS GLY ASP GLN ILE LEU LEU PRO GLN MET LEU SER
GLY LEU ASP GLU ARG GLU ASN ALA CYS PRO MET HIS VAL
ASP PHE SER ARG GLN LYS VAL SER HIS THR THR PHE GLY
HIS GLY SER HIS LEU CYS LEU GLY GLN HIS LEU ALA ARG
ARG GLU ILE ILE VAL THR LEU LYS GLU TRP LEU THR ARG
ILE PRO ASP PHE SER ILE ALA PRO GLY ALA GLN ILE GLN
HIS LYS SER GLY ILE VAL SER GLY VAL GLN ALA LEU PRO
LEU VAL TRP ASP PRO ALA THR THR LYS ALA VAL

# FIG. 4A



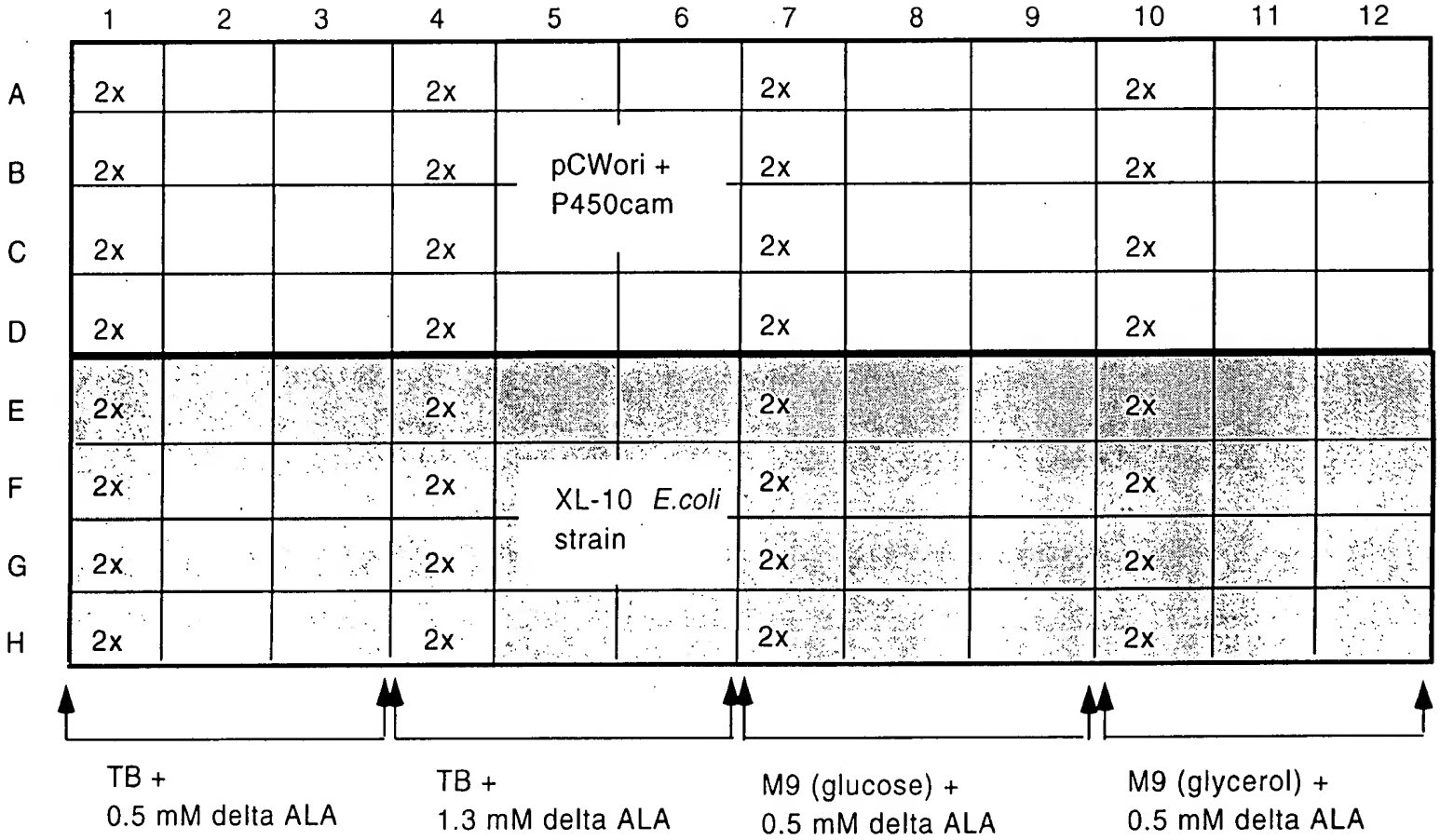

P450cam protein expression by adding 0.5-1 mM IPTG and 1 mM thaimine, 0.5 -1.3 mM delta-ALA, 0.5 ml trace element stock/10ml medium (total : 120 ul. vol)
Induction time : 24 hours
Induction temperature : 30 °C

# FIG. 4B



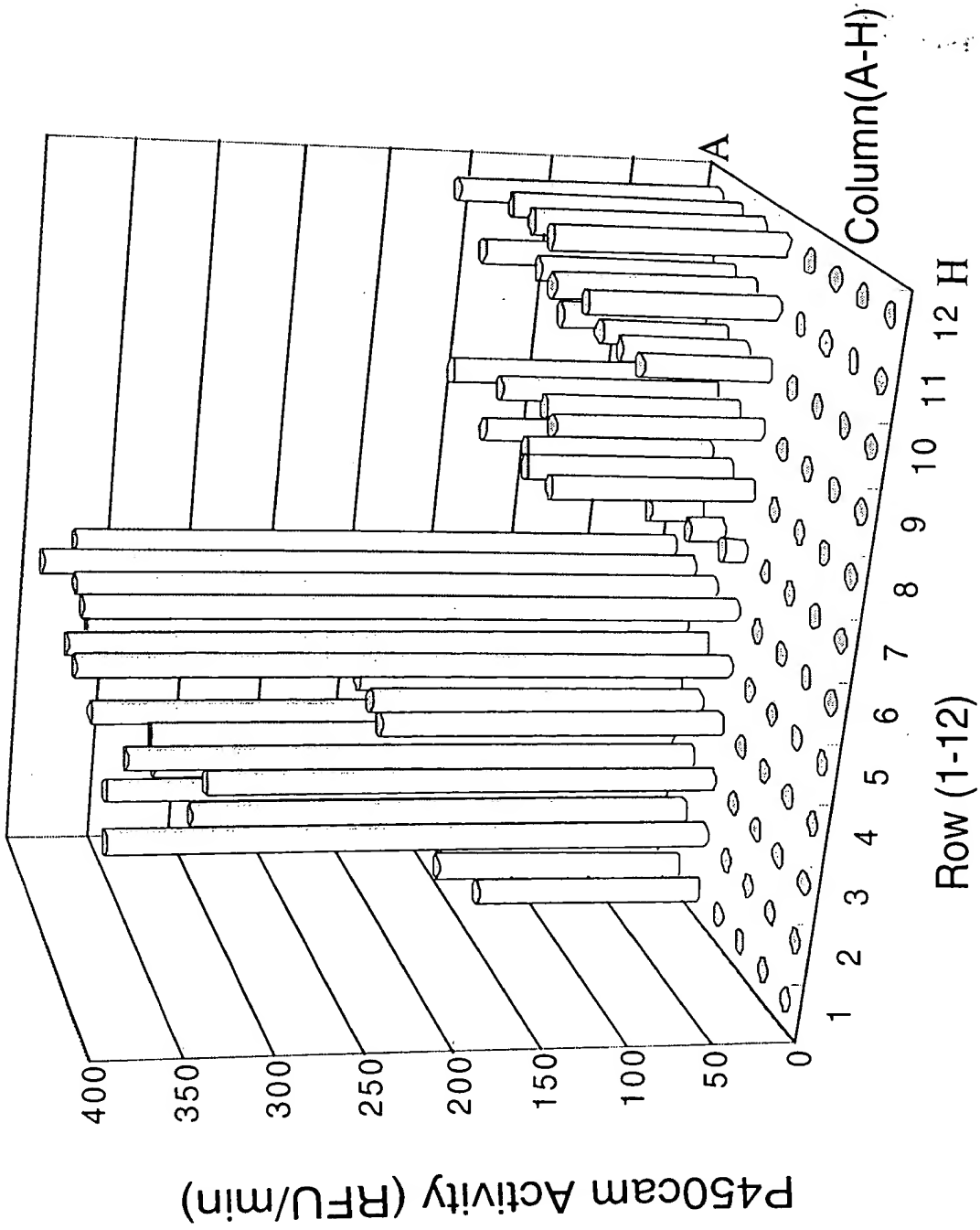

Reaction mixture (total 200 ul vol.):
- 50 ul culture broth
- 100 ul sodium dibasic phosphate buffer (50 mM, pH 9.0)
- 10 ul pure ethanol
- 20 ul naphthalene stock (1 g/13 ml ion pure ethanol)
- 10 ul hydrogen peroxide stock solution (100 mM)
- 10 ul horseradish peroxidase stock solution (1400 unitsa/10 ml)

# FIG. 5A




* 2x : 200 ul cultivation volume, others : 100 ul cultivation volume

FIG. 5B


P450cam Activity (RFU/min)
400
350
300
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12
Row (1-12)
A
H
Column(A-H)

# FIG. 6

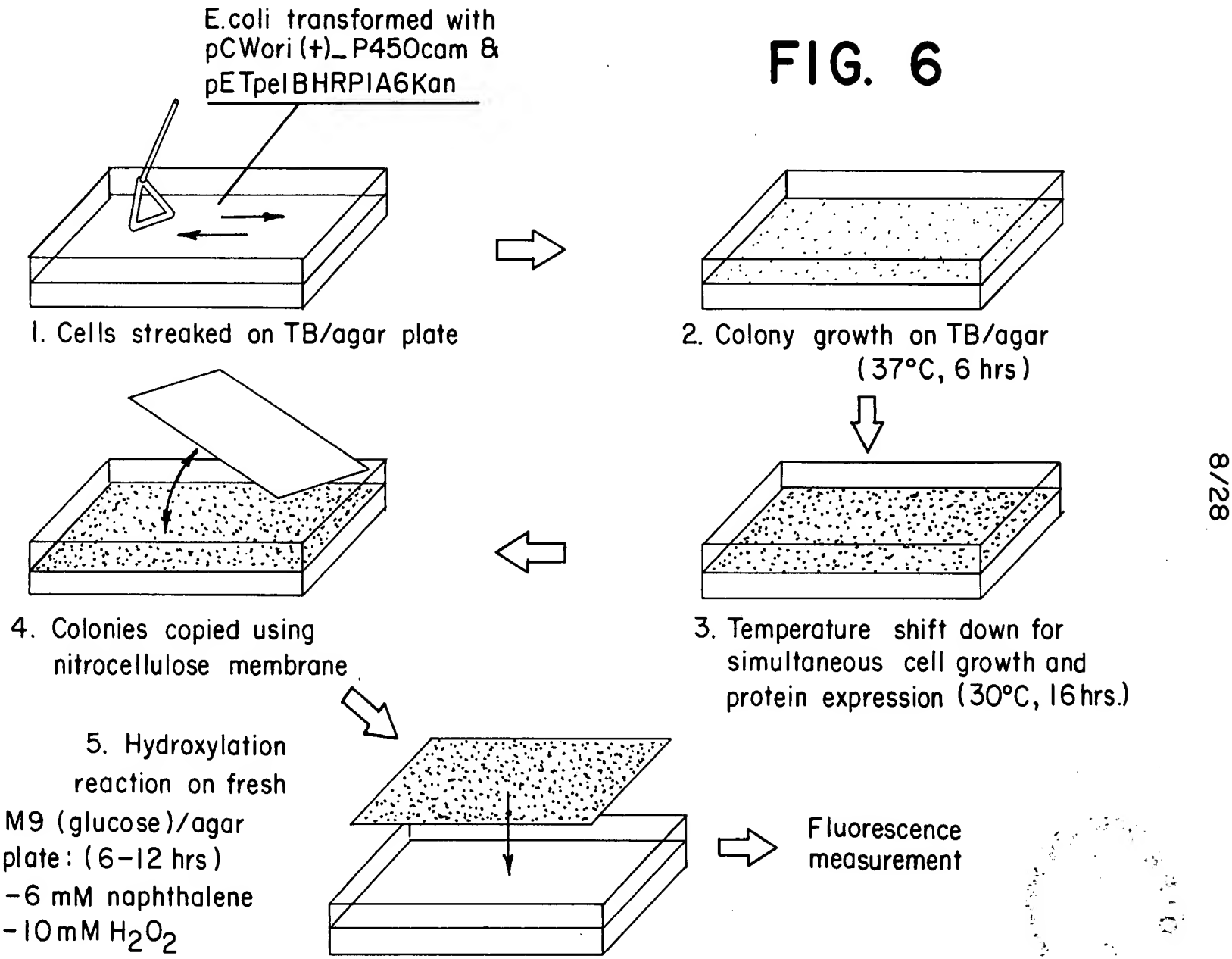

E.coli transformed with
pCWori(+)_P450cam &
pETpelBHRPIA6Kan
1. Cells streaked on TB/agar plate
2. Colony growth on TB/agar
(37°C, 6 hrs)
3. Temperature shift down for
simultaneous cell growth and
protein expression (30°C, 16hrs.)
4. Colonies copied using
nitrocellulose membrane
5. Hydroxylation
reaction on fresh
M9 (glucose)/agar
plate: (6-12 hrs)
-6 mM naphthalene
-10mM $H_2O_2$
Fluorescence
measurement

FIG. 7

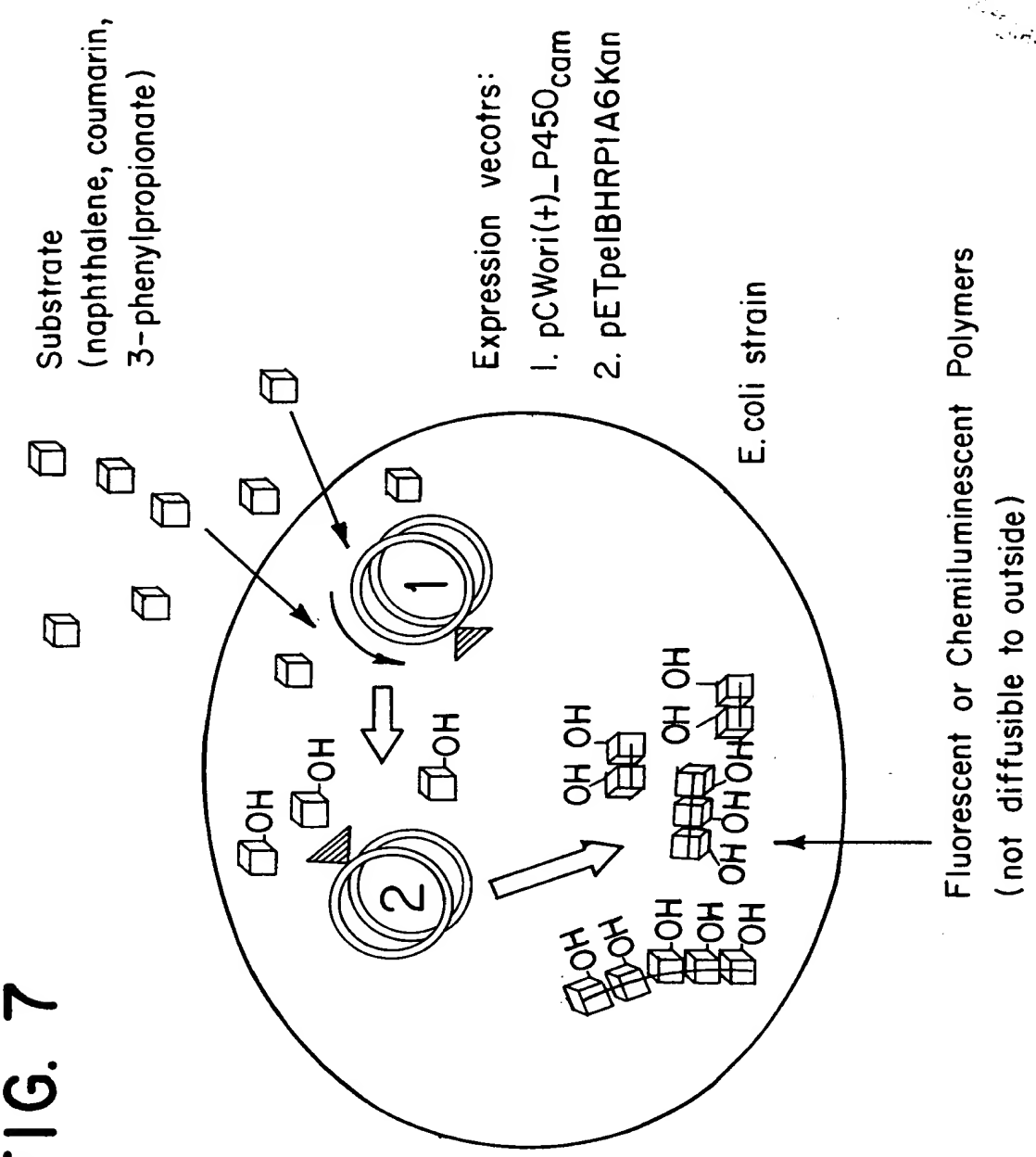
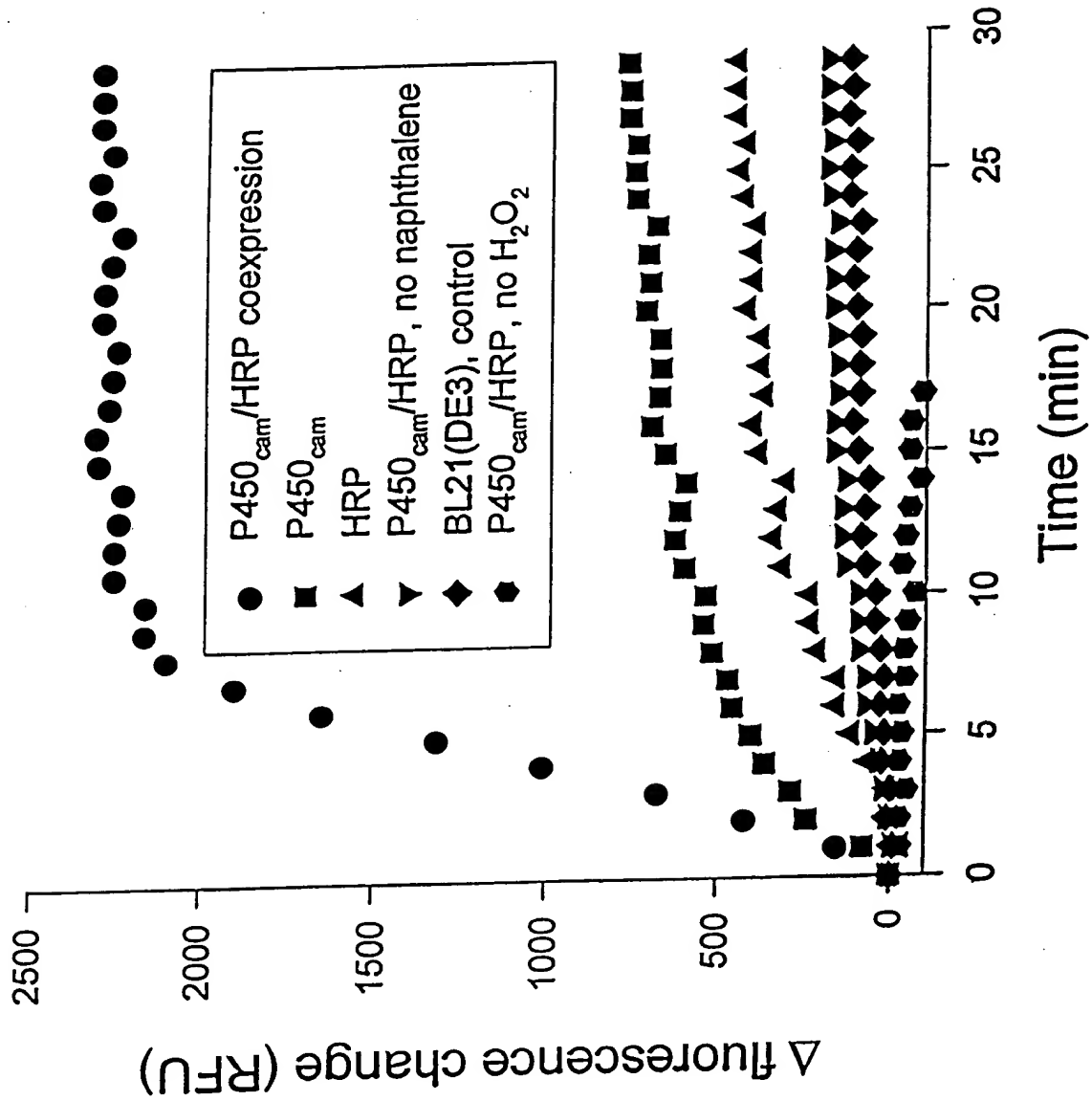

Substrate
(naphthalene, coumarin,
3-phenylpropionate)
Expression vecotrs:
1. pCWori(+)_P450cam
2. pETpeIBHRP1A6Kan
E. coli strain
Fluorescent or Chemiluminescent Polymers
(not diffusible to outside)
1
2
OH

FIG. 8


P450cam/HRP coexpression
P450cam
HRP
P450cam/HRP, no naphthalene
BL21(DE3), control
P450cam/HRP, no H2O2
Δ fluorescence change (RFU)
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Time (min)

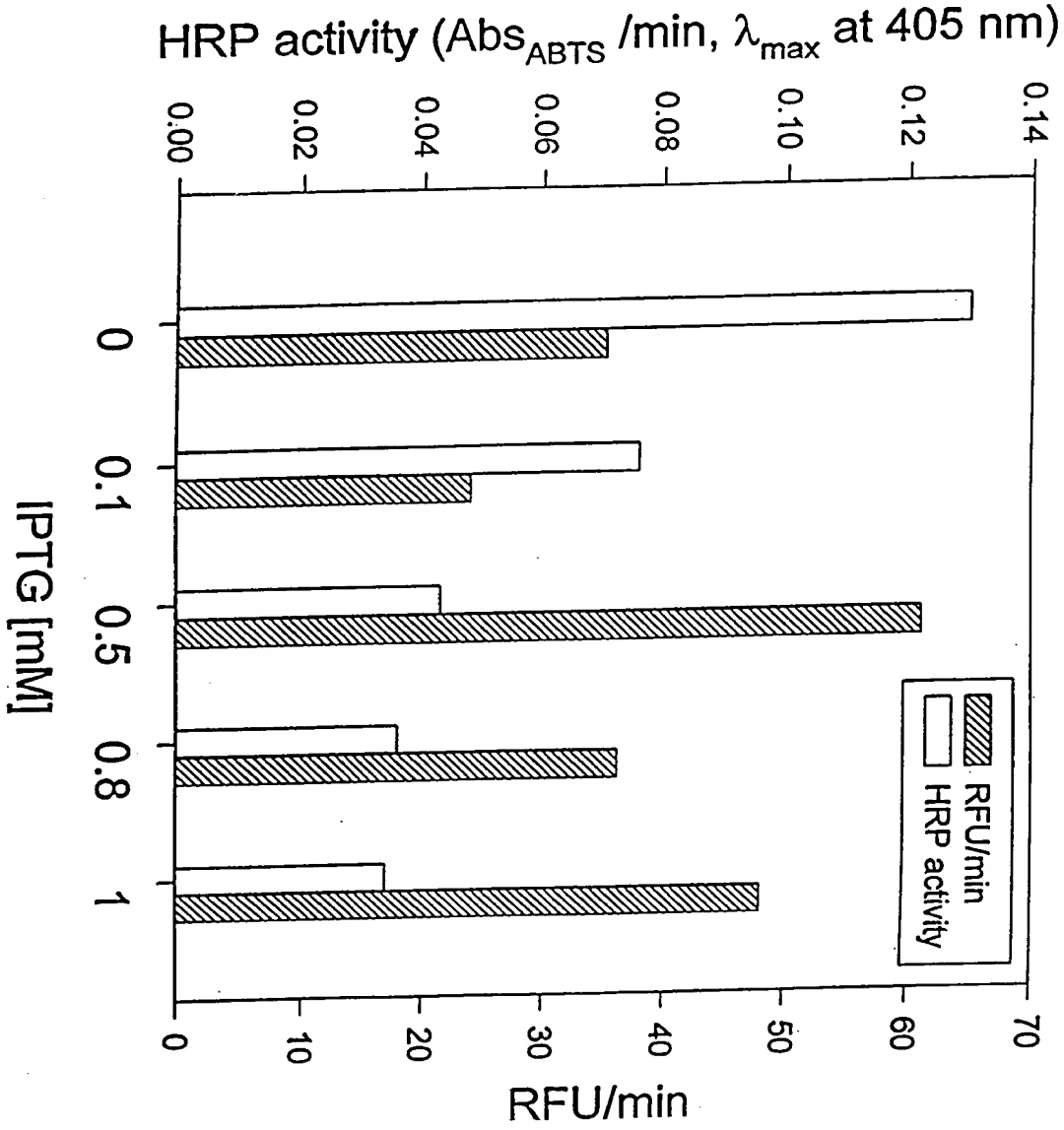

HRP activity (Abs$_{ABTS}$ /min, $\lambda_{max}$ at 405 nm)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
IPTG [mM]
0
0.1
0.5
0.8
1
RFU/min
HRP activity
0
10
20
30
40
50
60
70
RFU/min

FIG. 9

FIG. 10

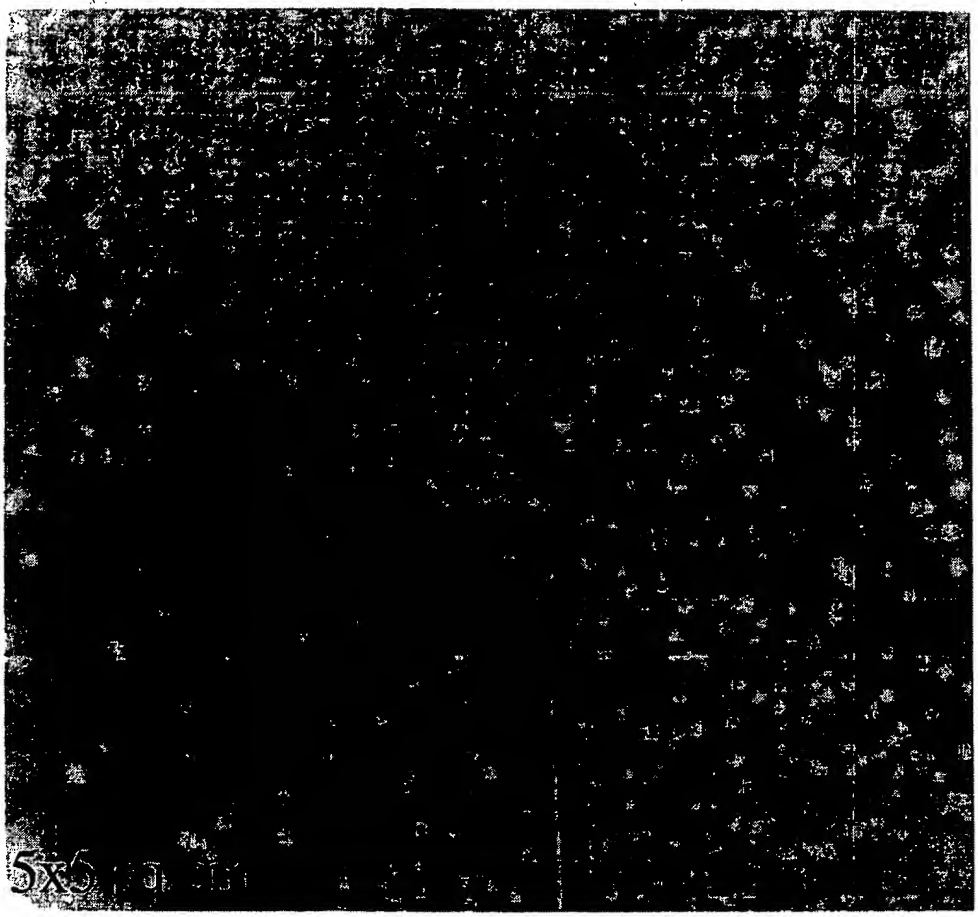


FIG. 12A

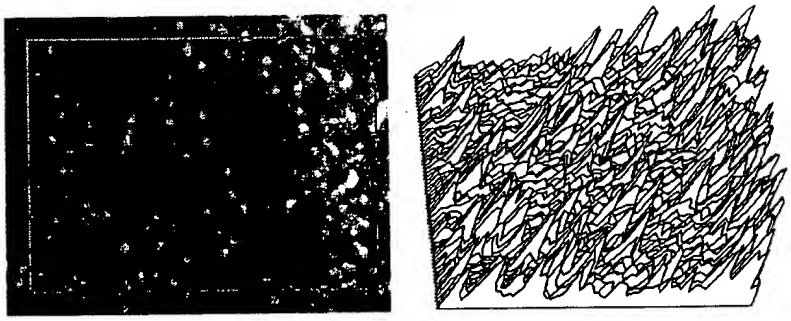



FIG. 12B

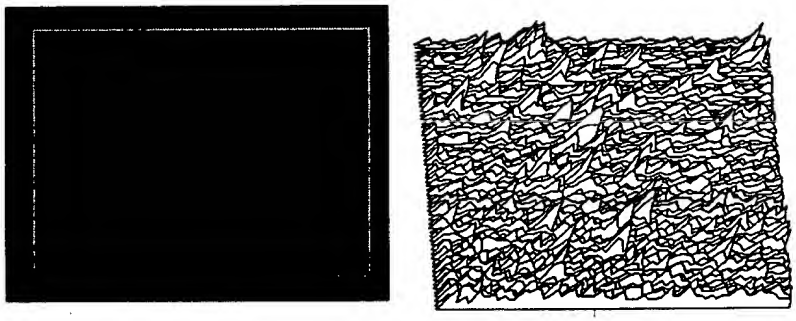


FIG. 11A

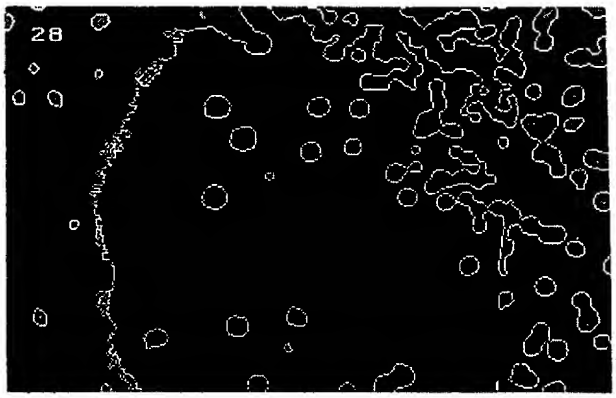



FIG. 11B

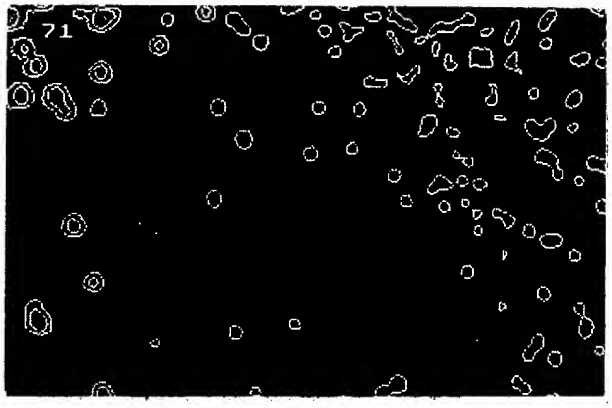



FIG. 11C

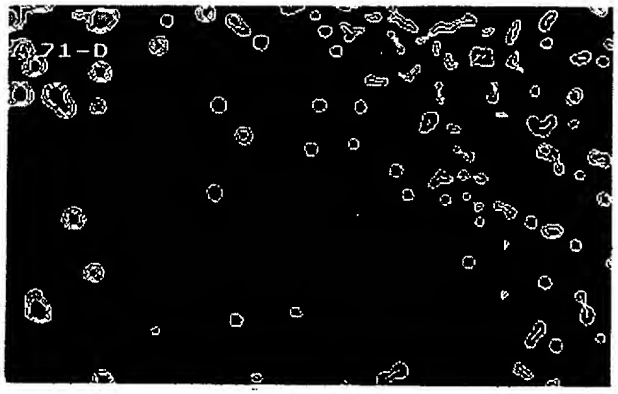



FIG. 11D

FIG. 12C

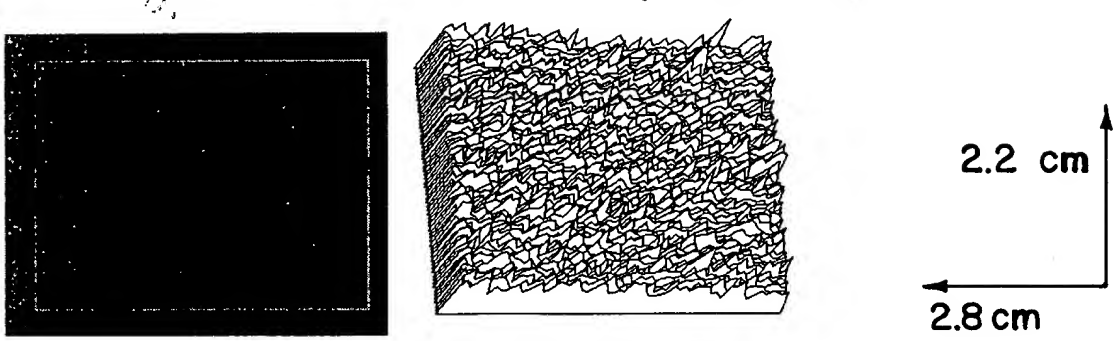



FIG. 12D

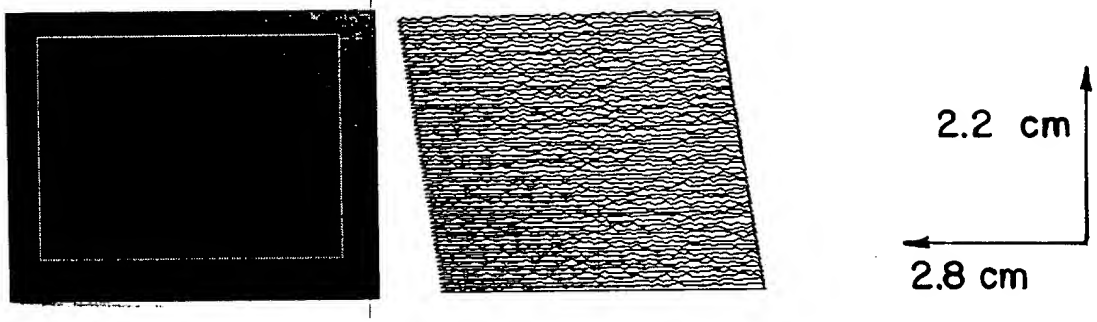



FIG. 12E

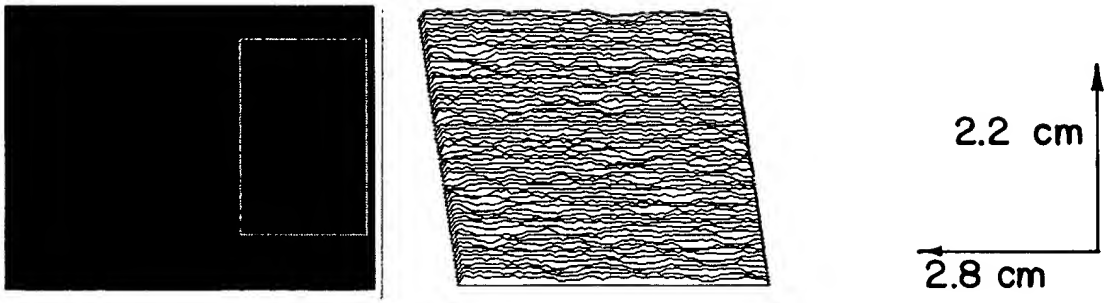



FIG. 12F

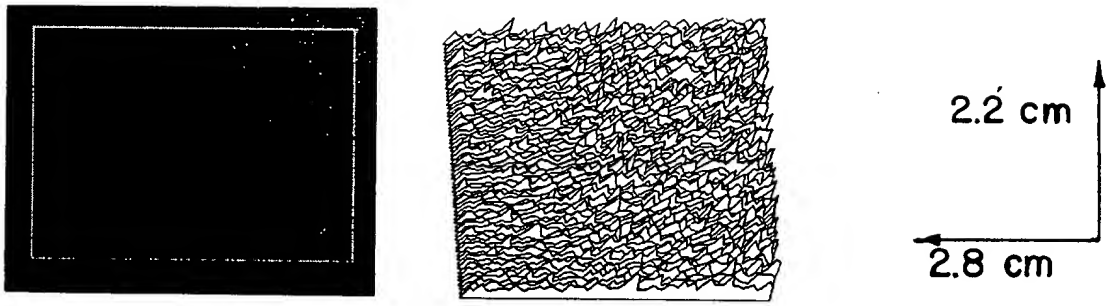


FIG. 13

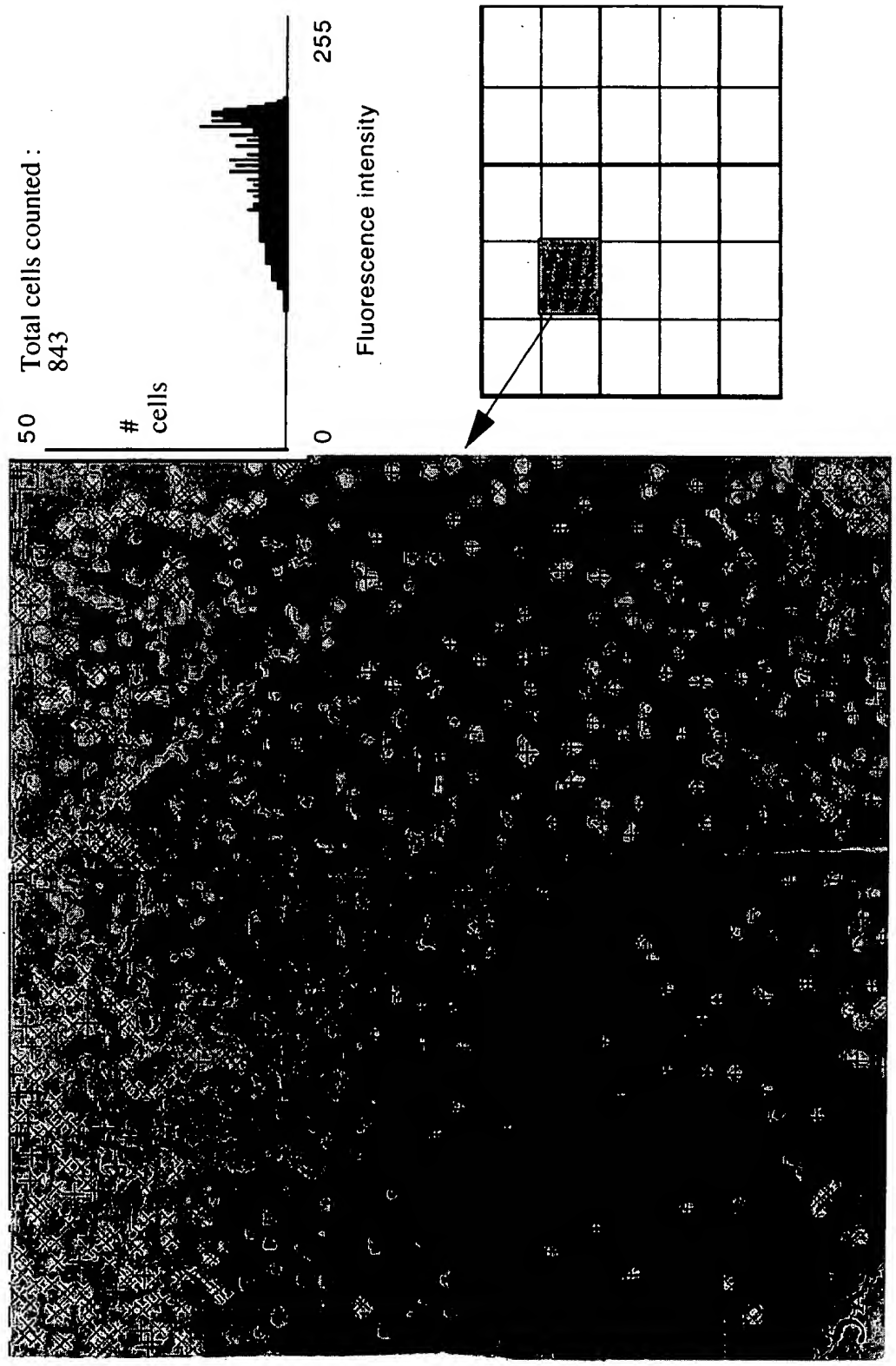

Total cells counted :
843
# cells
50
0
255
Fluorescence intensity

FIG. 14A

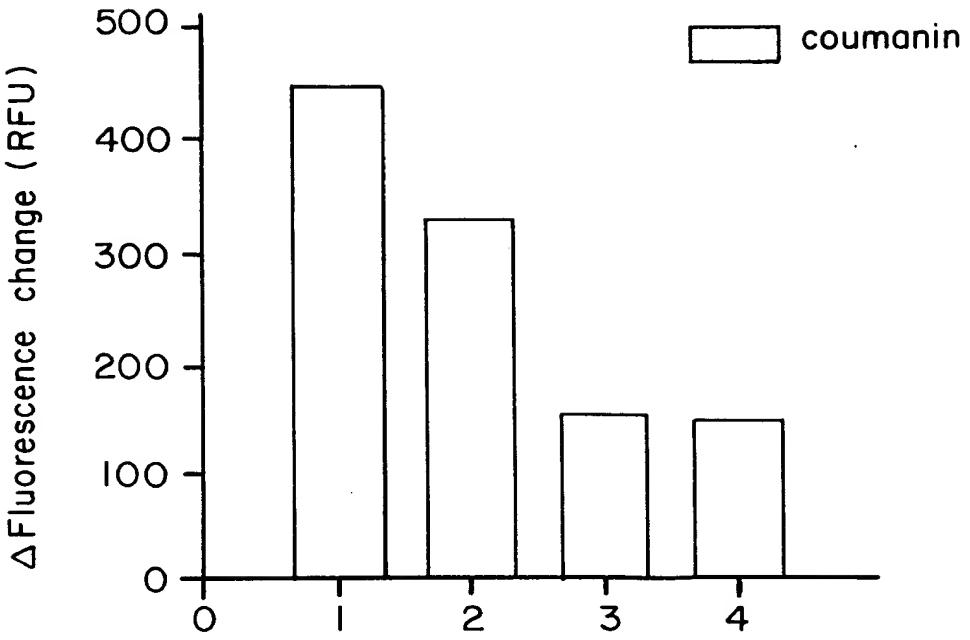
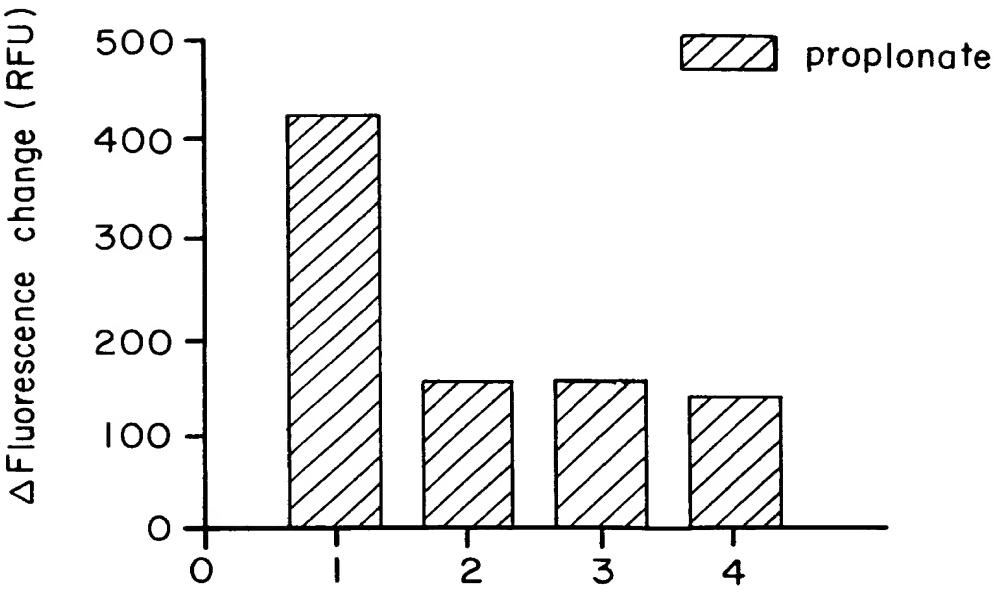
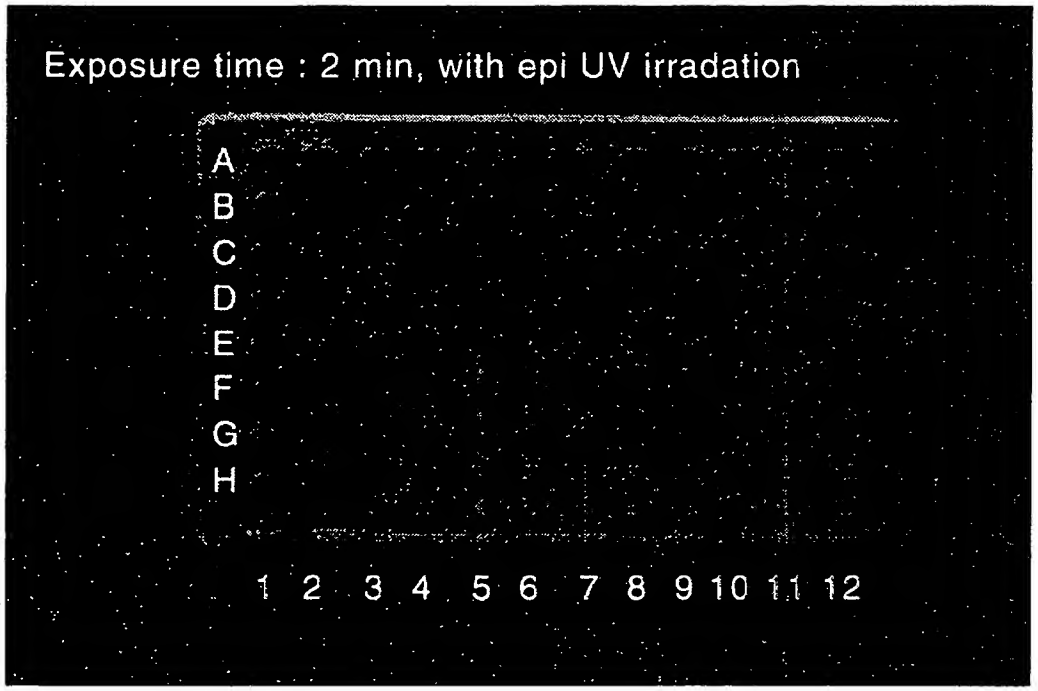



FIG. 14B



# FIG. 15A



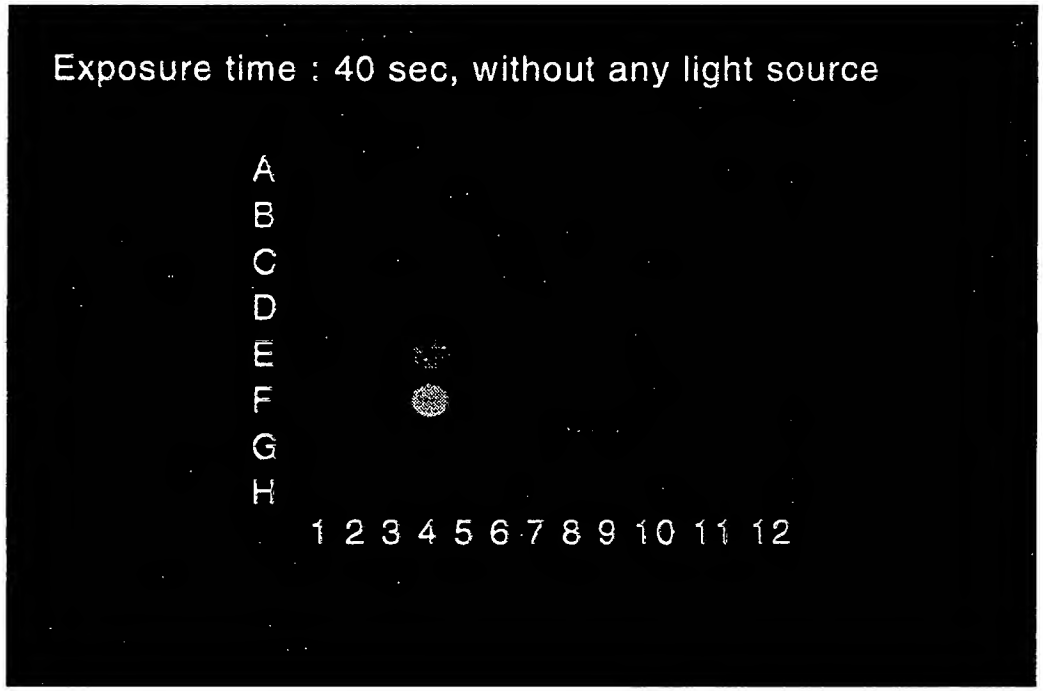

Row:

E: 60uM luminol+0.5 mM PPP
F: 120 uM luminol+0.5 mM PPP
G: 60 uM luminol
H: 120 uM luminol

Column:

4: P450cam/HRP1A6 in BL21 (DE3)
5: P450cam in BL21 (DE3)
6: HRP1A6 in BL21 (DE3)
7: Host strain, BL21 (DE3)

FIG. 15B




Light Emission Values

E4: 51 ILDV
F4: 98 ILDV
G4: 0.2 ILDV
H4: 1 ILDV

Others: <0.1 ILDV

# FIG. 17

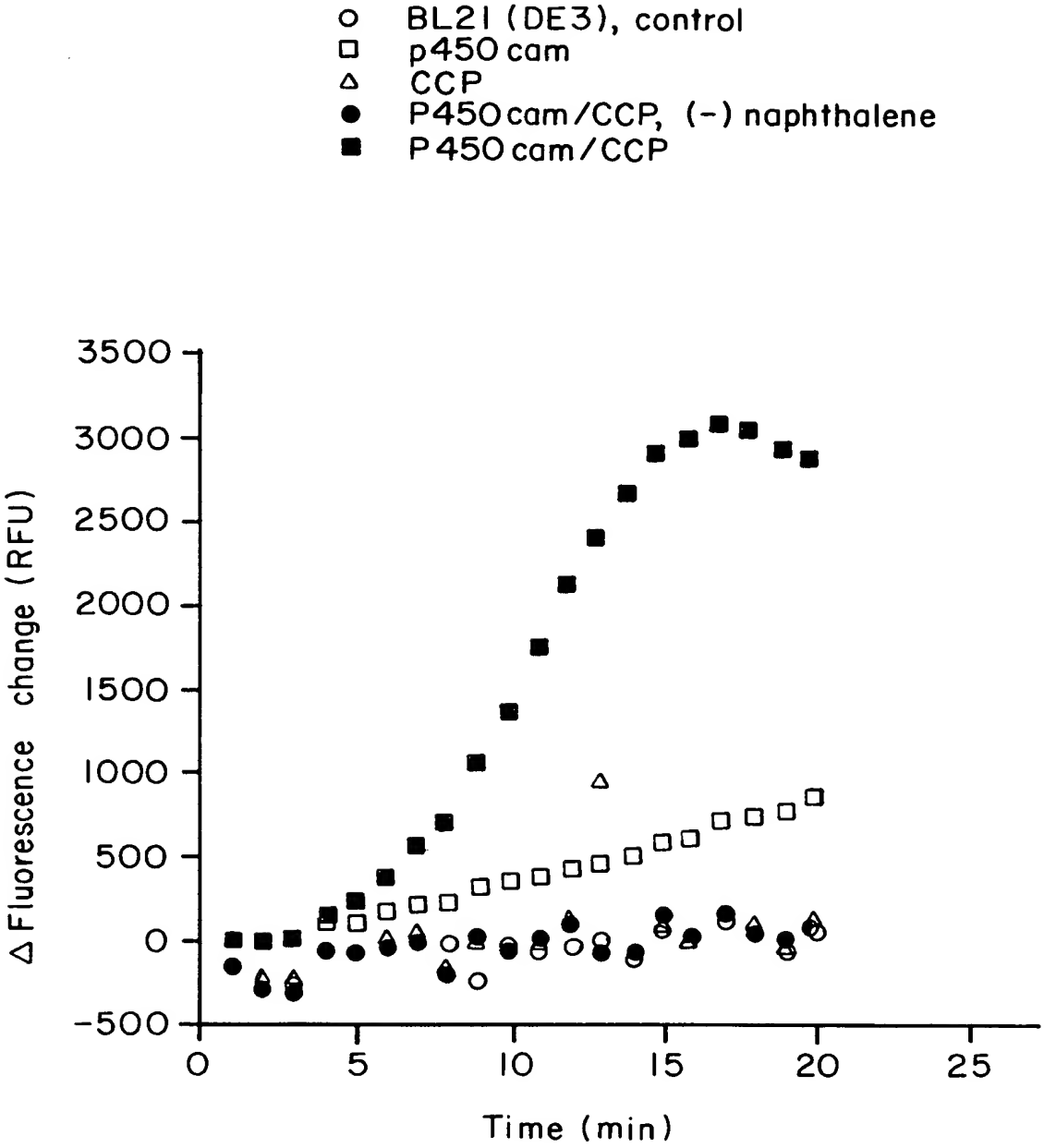

BL21 (DE3), control
p450 cam
CCP
P450 cam/CCP, (-) naphthalene
P450 cam/CCP
Δ Fluorescence change (RFU)
3500
3000
2500
2000
1500
1000
500
0
-500
0
5
10
15
20
25
Time (min)

FIG. 18

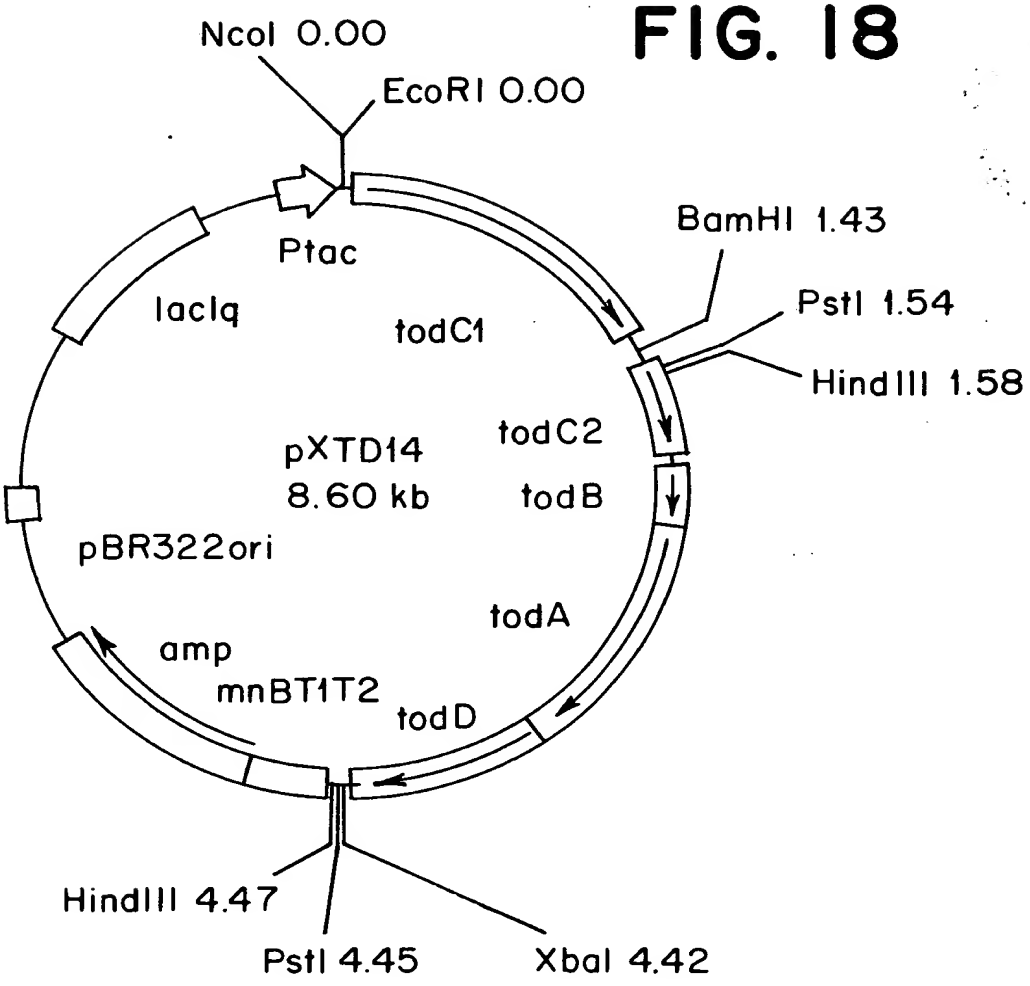

NcoI 0.00
EcoRI 0.00
BamHI 1.43
PstI 1.54
HindIII 1.58
Ptac
lacIq
todC1
todC2
pXTD14
8.60 kb
todB
pBR322ori
todA
amp
mnBT1T2
todD
HindIII 4.47
PstI 4.45
XbaI 4.42

FIG. 21

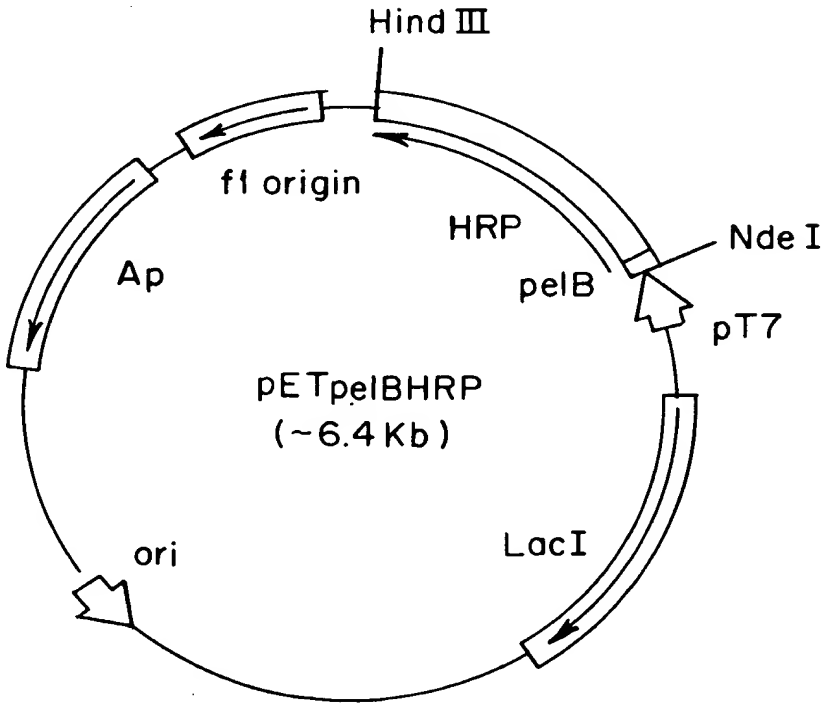

Hind III
f1 origin
HRP
Nde I
Ap
pelB
pT7
pETpelBHRP
(~6.4 Kb)
ori
LacI

FIG. 19A

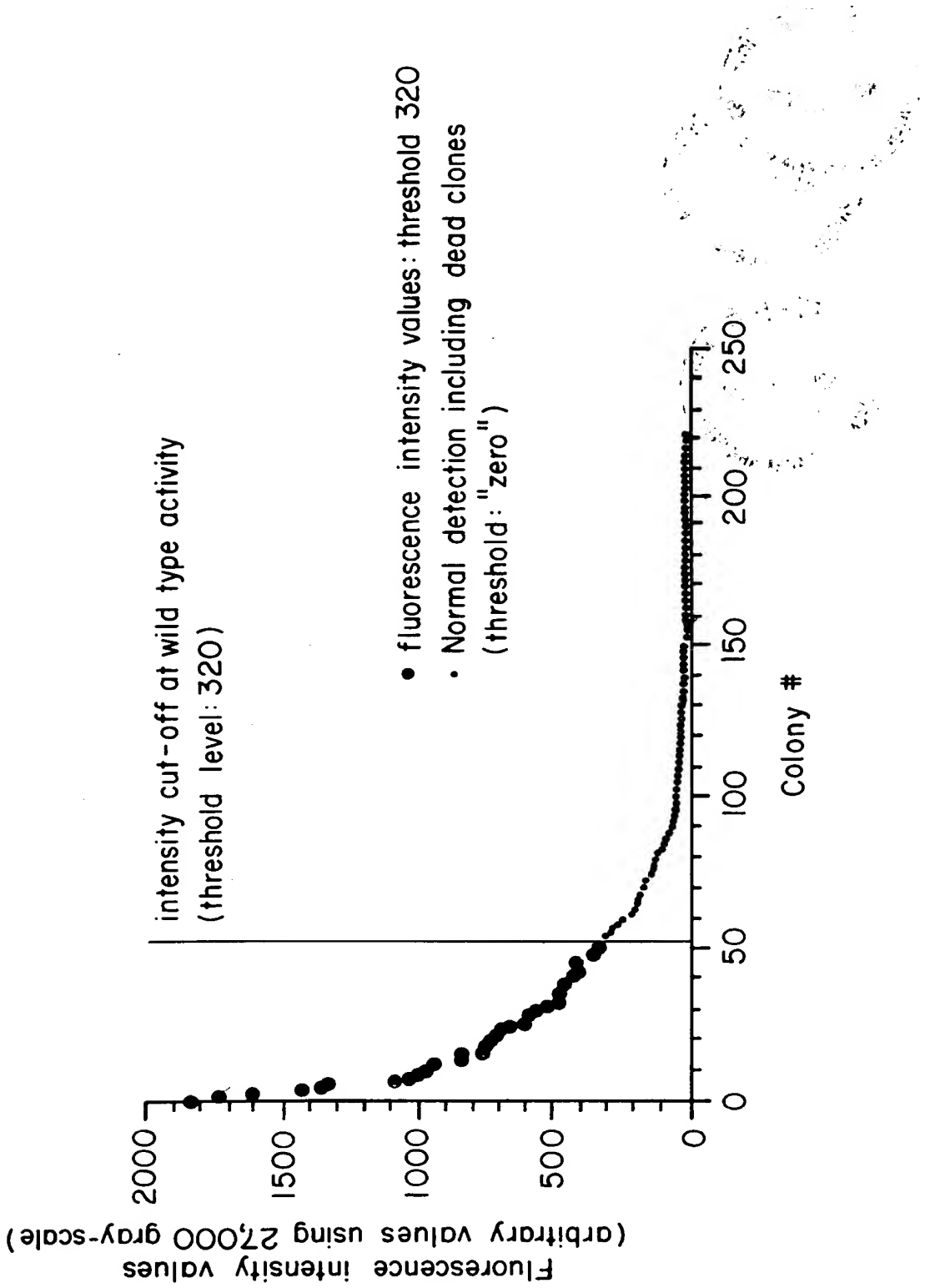

Fluorescence intensity values
(arbitrary values using 27,000 gray-scale)
2000
1500
1000
500
0
0
50
100
150
200
250
Colony #
intensity cut-off at wild type activity
(threshold level: 320)
• fluorescence intensity values: threshold 320
• Normal detection including dead clones
(threshold: "zero")

FIG. 19B

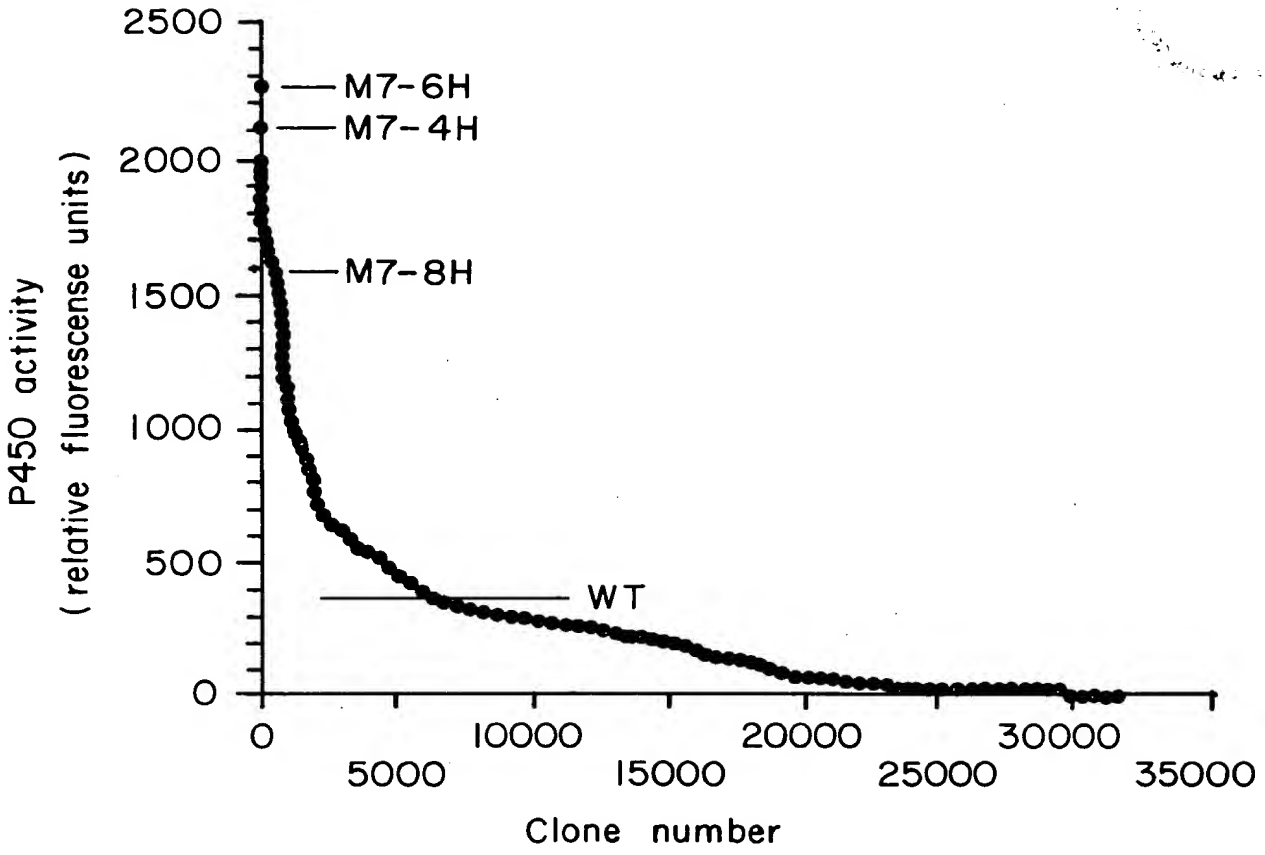



FIG. 19C

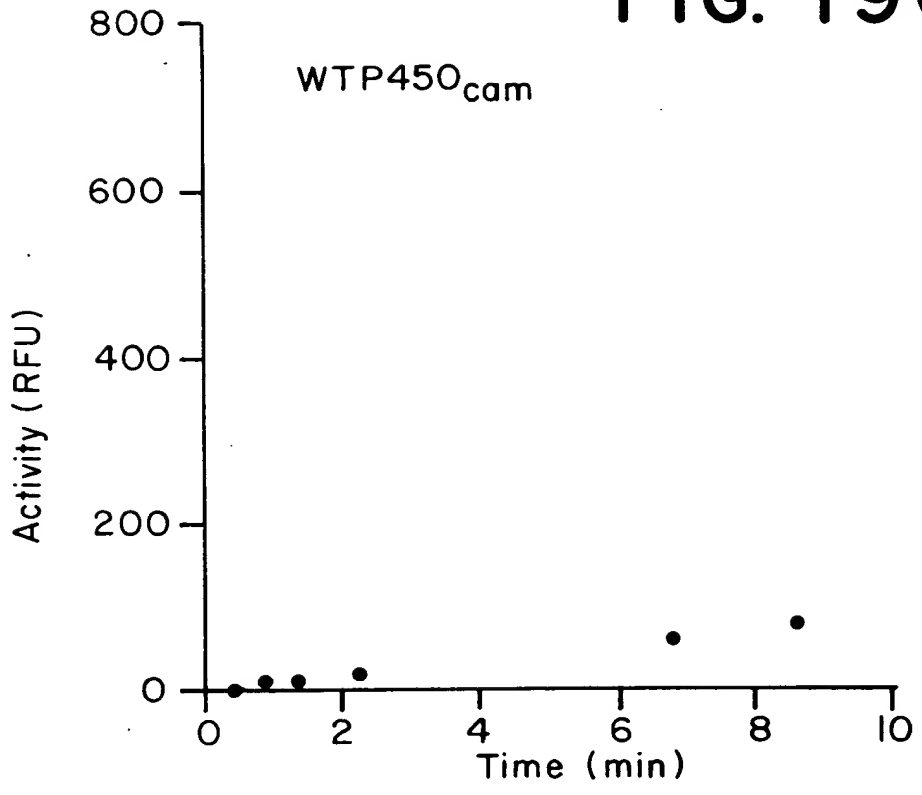
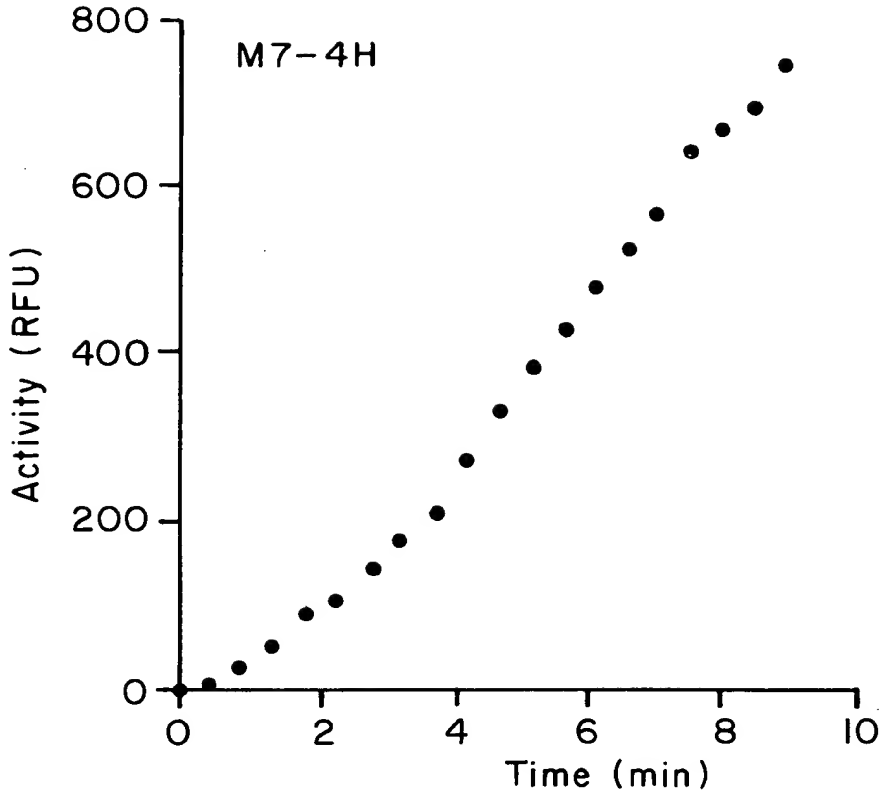


FIG. 19D



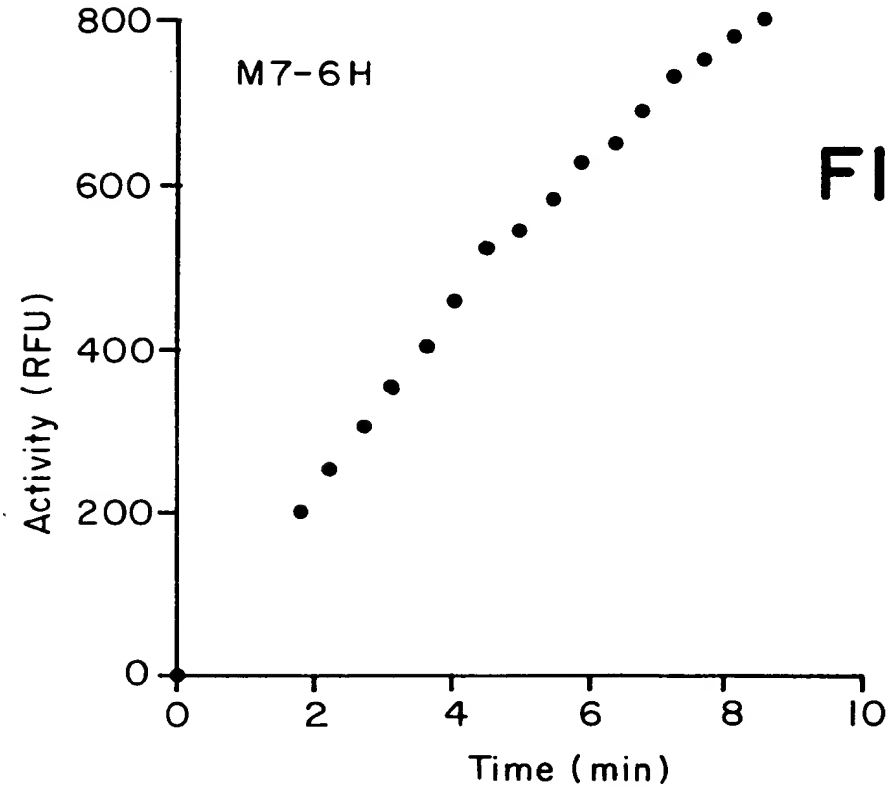




FIG. 19E

FIG. 19F

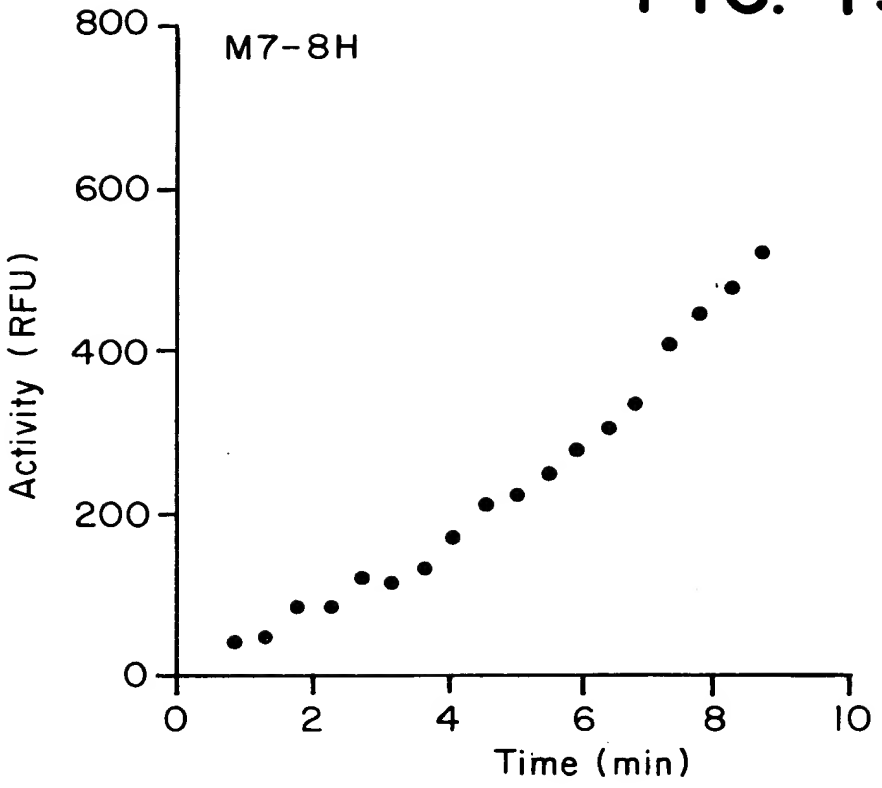
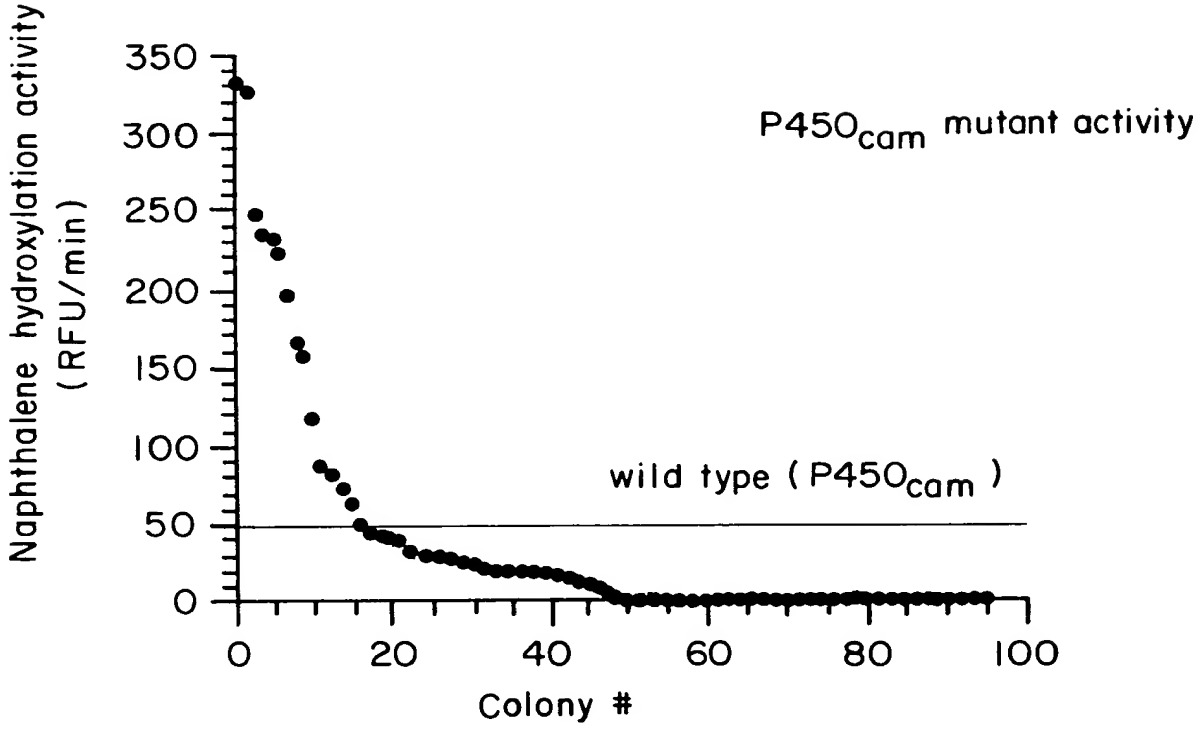



FIG. 20



# FIG. 22

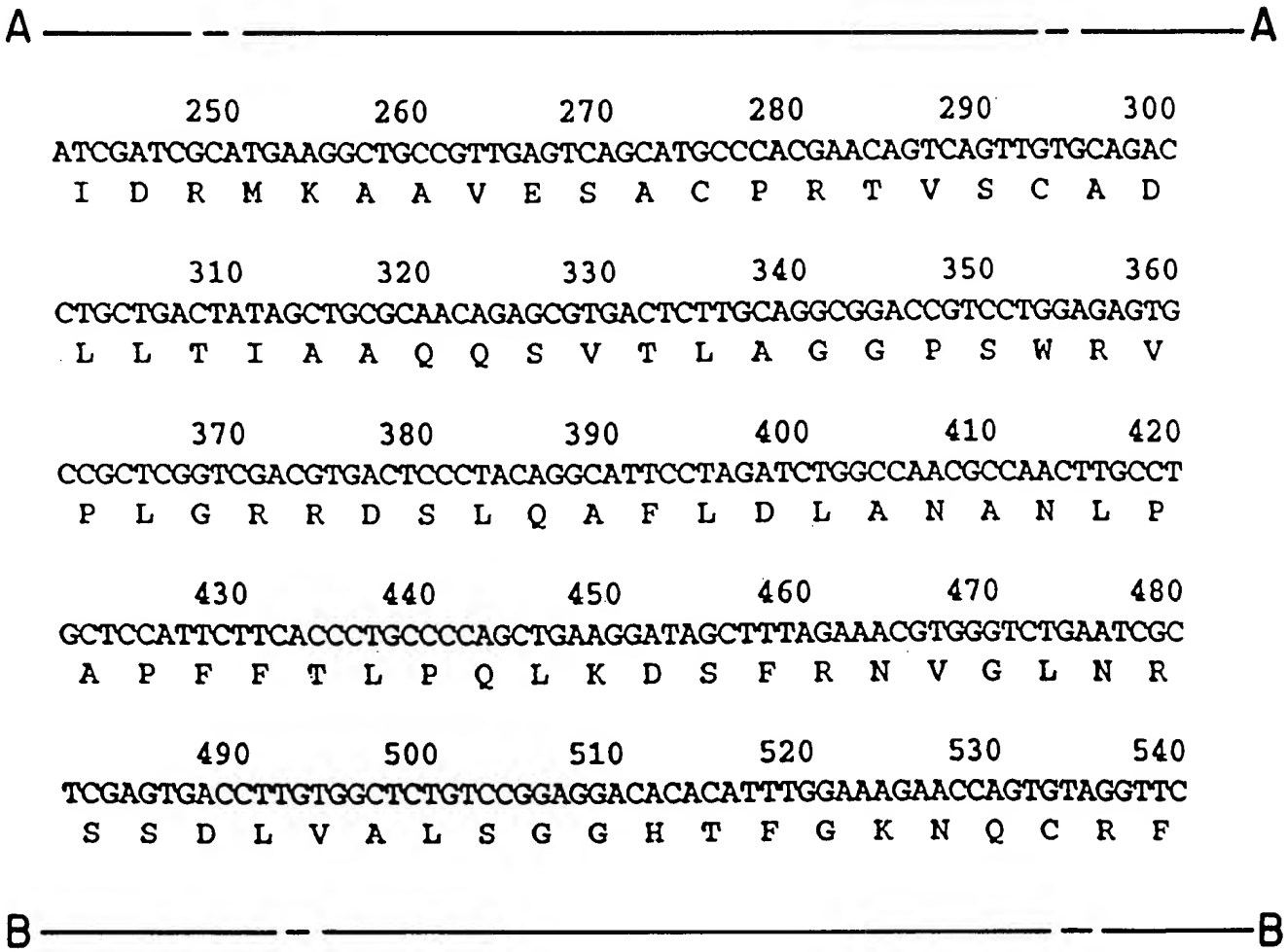
ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCT GCC CAA CCA GCC ATG GCC
Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala Met Ala

# FIG. 23A

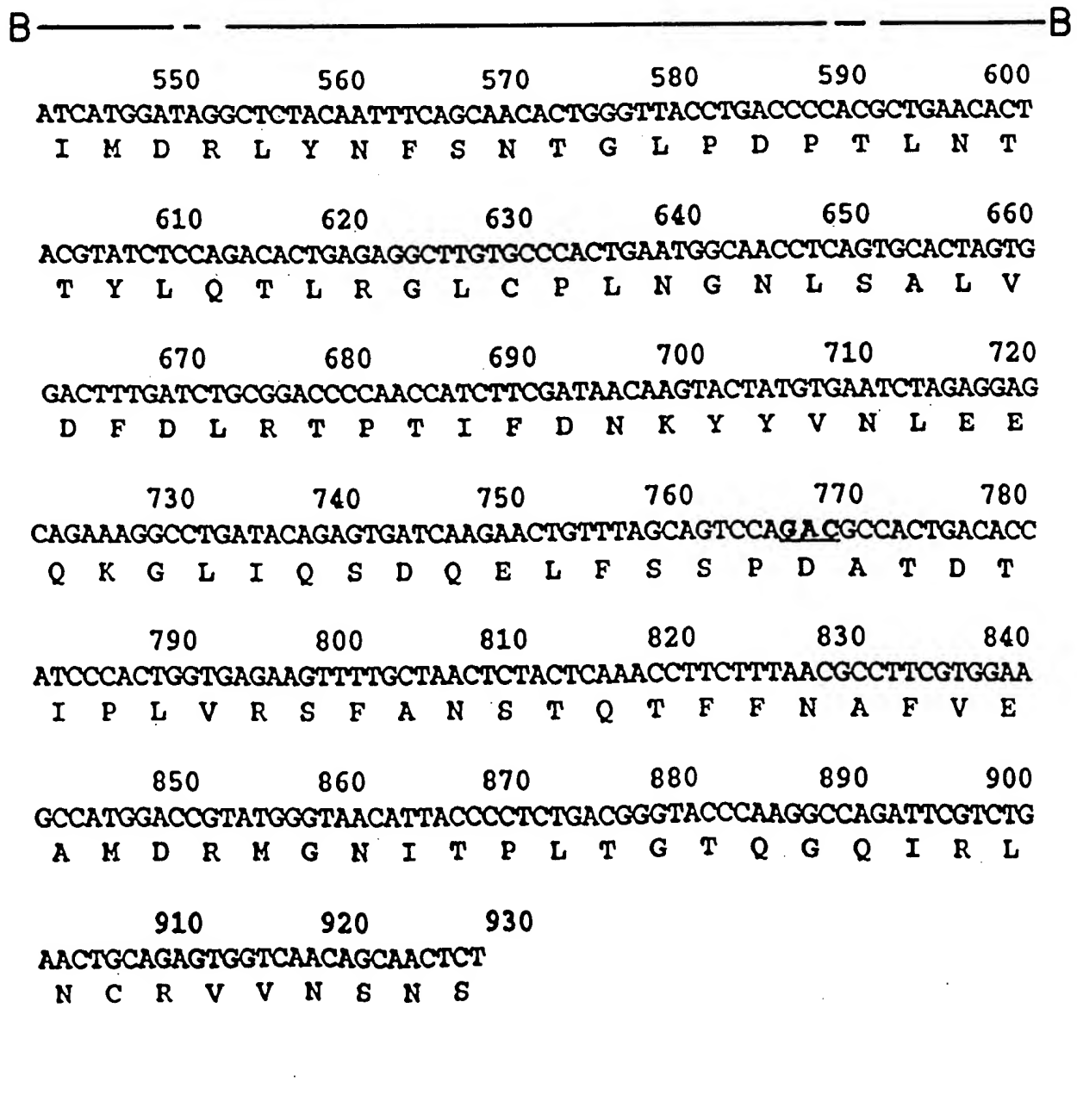
```
          10         20         30         40         50         60
ATGCAGTTAACCCCTACATTCTACGACAATAGCTGTCCCAACGTGTCCAACATCGTTCGC

 M  Q  L  T  P  T  F  Y  D  N  S  C  P  N  V  S  N  I  V  R

          70         80         90        100        110        120
GACACAATCGTCAACGAGCTCAGATCCGATCCCAGGATCGCTGCTTCAATATTACGTCTG
 D  T  I  V  N  E  L  R  S  D  P  R  I  A  A  S  I  L  R  L

         130        140        150        160        170        180
CACTTCCATGACTGCTTCGTGAATGGTTGCGACGCTAGCATATTACTGGACAACACCACC
 H  F  H  D  C  F  V  N  G  C  D  A  S  I  L  L  D  N  T  T

         190        200        210        220        230        240
AGTTTCCGCACTGAAAAGGATGCATTCGGGAACGCTAACAGCGCCAGGGGCTTTCCAGTG
 S  F  R  T  E  K  D  A  F  G  N  A  N  S  A  R  G  F  P  V
```

A——— - ——————————————————————— - ———A

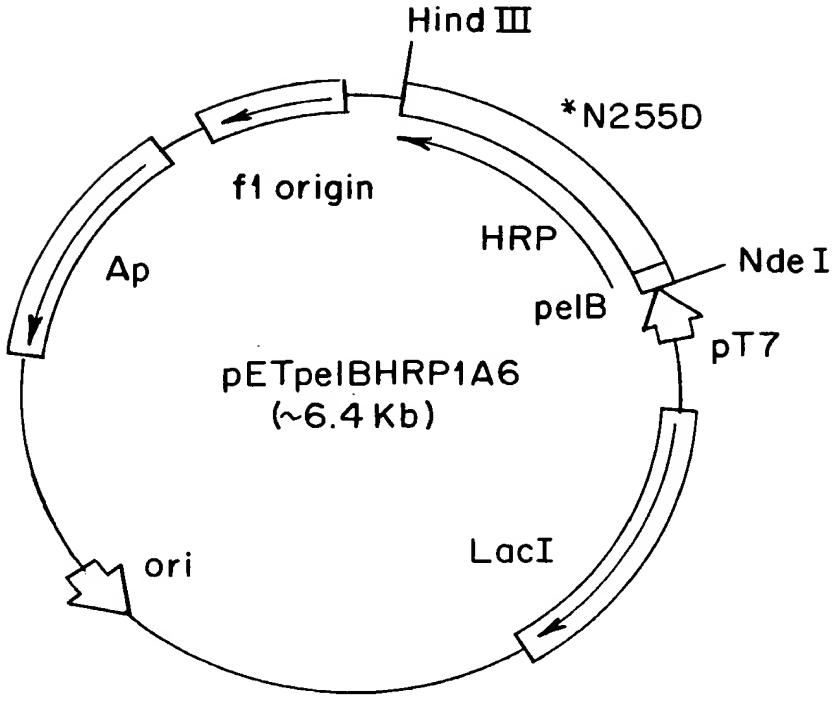
# FIG. 23B


A
A
250 260 270 280 290 300
ATCGATCGCATGAAGGCTGCCGTTGAGTCAGCATGCCCACGAACAGTCAGTTGTGCAGAC
I D R M K A A V E S A C P R T V S C A D
310 320 330 340 350 360
CTGCTGACTATAGCTGCGCAACAGAGCGTGACTCTTGCAGGCGGACCGTCCTGGAGAGTG
L L T I A A Q Q S V T L A G G P S W R V
370 380 390 400 410 420
CCGCTCGGTCGACGTGACTCCCTACAGGCATTCCTAGATCTGGCCAACGCCAACTTGCCT
P L G R R D S L Q A F L D L A N A N L P
430 440 450 460 470 480
GCTCCATTCTTCACCCTGCCCCAGCTGAAGGATAGCTTTAGAAACGTGGGTCTGAATCGC
A P F F T L P Q L K D S F R N V G L N R
490 500 510 520 530 540
TCGAGTGACCTTGTGGCTCTGTCCGGAGGACACACATTTGGAAAGAACCAGTGTAGGTTC
S S D L V A L S G G H T F G K N Q C R F
B
B

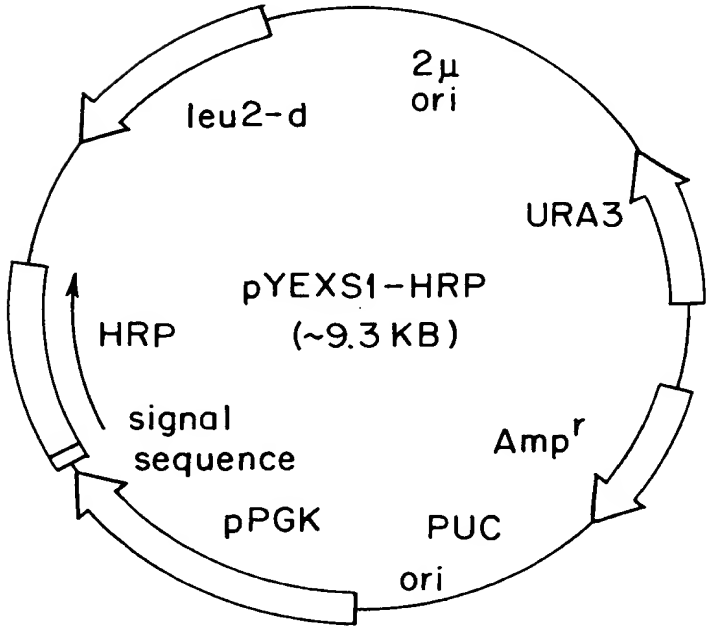
# FIG. 23C


B
B
550 560 570 580 590 600
ATCATGGATAGGCTCTACAATTTCAGCAACACTGGGTTACCTGACCCCACGCTGAACACT
I M D R L Y N F S N T G L P D P T L N T
610 620 630 640 650 660
ACGTATCTCCAGACACTGAGAGGCTTGTGCCCACTGAATGGCAACCTCAGTGCACTAGTG
T Y L Q T L R G L C P L N G N L S A L V
670 680 690 700 710 720
GACTTTGATCTGCGGACCCCAACCATCTTCGATAACAAGTACTATGTGAATCTAGAGGAG
D F D L R T P T I F D N K Y Y V N L E E
730 740 750 760 770 780
CAGAAAGGCCTGATACAGAGTGATCAAGAACTGTTTAGCAGTCCAGACGCCACTGACACC
Q K G L I Q S D Q E L F S S P D A T D T
790 800 810 820 830 840
ATCCCACTGGTGAGAAGTTTTGCTAACTCTACTCAAACCTTCTTTAACGCCTTCGTGGAA
I P L V R S F A N S T Q T F F N A F V E
850 860 870 880 890 900
GCCATGGACCGTATGGGTAACATTACCCCTCTGACGGGTACCCAAGGCCAGATTCGTCTG
A M D R M G N I T P L T G T Q G Q I R L
910 920 930
AACTGCAGAGTGGTCAACAGCAACTCT
N C R V V N S N S

# FIG. 24


Hind III
*N255D
f1 origin
HRP
Ap
Nde I
pelB
pT7
pETpelBHRP1A6
(~6.4 Kb)
ori
LacI

# FIG. 25


2μ
ori
leu2-d
URA3
pYEXS1-HRP
(~9.3 KB)
HRP
signal
sequence
Amp^r
pPGK
PUC
ori